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Fluorescent Proteins 101

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INTRODUCTION TO FLUORESCENT PROTEINS 101

By Tyler J. Ford and The Addgene Team | October, 2016

Dear Readers,

Before compiling this eBook, I knew that fluorescent proteins were amazing tools, but I didn't quite grasp just how awesome they are until fellow Addgenie Mary Gearing and I began planning out the full eBook.

Fluorescent proteins have truly revolutionized molecular biology studies. We now take it for granted that you can quickly label cells and proteins with a wide array of colors - keeping in mind a few caveats. In addition, creative new applications, like using GFP to activate gene expression, allow fluorescent proteins to play a role in many different types of experiments. While we couldn't discuss every application of fluorescent proteins, we hope that this eBook will provide you with the basics for your next experiment and maybe inspire you to come up with a new application of your own.

Before we get to the science, we'd like to thank our many fluorescent protein depositors and researchers, and, in particular, blue flame depositors Roger Tsien and Michael Davidson, without whom much of the work in this eBook couldn't be performed. In addition, we'd like to thank our many writers (Addgenies and guests alike) who have worked hard to bring you all this great content in an easily digestible form. Finally, we'd like to thank you, our readers, who will help further expand the world of fluorescent proteins and whose thoughtful feedback will help improve the future editions of this eBook.

Please read on to dive into the world of fluorescent proteins and, as always, let us know if you have any questions or comments by shooting us an email at blog.addgene.org.

Happy Reading!

Tyler J. Ford and the Addgene Team


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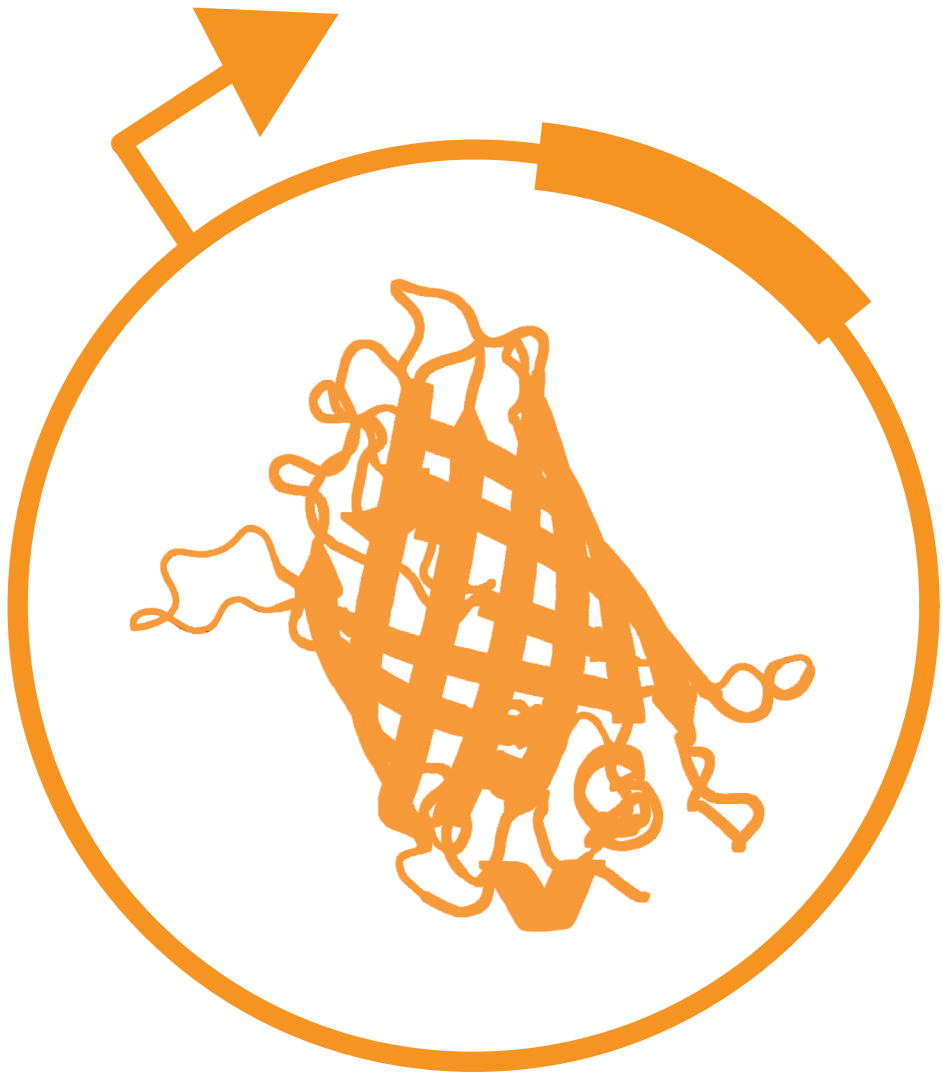


THE HISTORY OF FLUORESCENT PROTEINS

By A. Max Juchheim, Addgene | July, 2017

Luminescent molecules are very useful tools because we can easily detect and measure the light they emit. Proteins that give off light include chemiluminescent proteins, like luciferases, as well as fluorescent ones, like Green Fluorescent Protein (GFP). These molecules occur naturally in bioluminescent organisms, but their real power lies in the clever ways sceintists have adapted them for use in the laboratory.

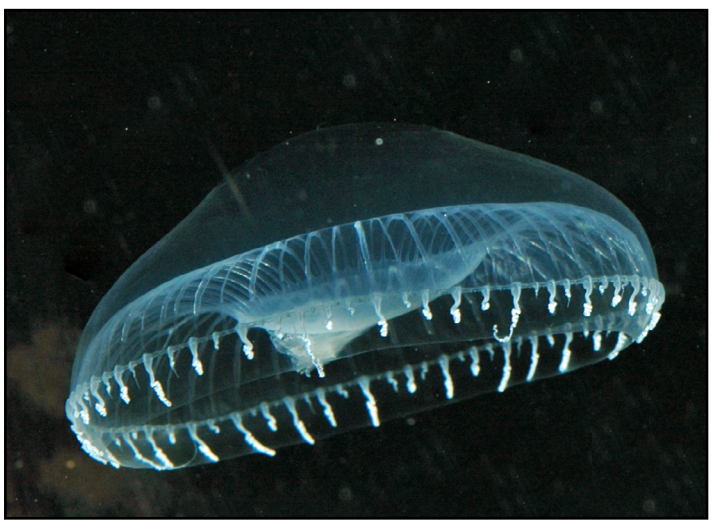


Figure 1: Aequorea victoria. Image Attribution: Sierra Blakely.

The Discovery of GFP

The story of how fluorescent proteins like GFP came to be used as research tools is particularly interesting. Although humans have known about bioluminescent organisms and their characteristic radiance for some time – there are records describing them dating back to the first century A.D. – the first time anyone truly examined what caused this glowing phenomenon wasn't until much later when, in the 1960s, Osamu Shimomura studied the bioluminescent properties observed in the crystal jellyfish *Aequorea victoria* (Figure 1) in molecular detail.

On the underside of the "umbrella" of *A. victoria* there is a ring that glows with a faint green light. To investigate this green glimmer, Shimomura collected many, many jellyfish specimens from Puget Sound in the Pacific Ocean, off of the coast of Washington state. Using these samples, he was able to isolate two proteins from the jellyfish's photoorgans; the first, which he called aequorin, gives off a faint blue light when it binds calcium ions, and the second, which we now call GFP, absorbs that blue light and glows green.

In the late 1980s, another researcher, Douglas Prasher, got the idea that this new green glowing protein could be used to measure gene transcription, as well as to track protein localization. And as it turns out, he was right! He began to study the *A. victoria* gene responsible for encoding GFP, and, in 1992, he reported its sequence. Soon after that, in 1994, Prasher's collaborator Martin Chalfie expressed GFP in exogenous organisms (*E. coli*, and later *C. elegans*) for the first time.

After Chalfie's work, scientists really started to take notice of GFP, and began to realize its potential as a research tool. Soon, the number of people studying it grew. In particular, studies in the laboratory of UCSD biochemist Roger Tsien resulted in much of our current mechanistic understanding of GFP's function. His lab described the protein's structure and also discovered many mutations that alter and improve its function and shift its emission spectrum.

New Fluorescent Proteins with New Colors

Around the same time, researcher Sergey Lukyanov realized that, although they are not bioluminescent, some types of corals and anemones are vibrantly colored. He hypothesized that GFP-like proteins could be responsible and began studying these marine organisms to find the culprits. He even obtained samples of certain ones from local pet shop aquariums in Moscow where he lived! His hunch paid off, and he was able to report new fluorophores like DsRed from *Discosoma sp.* and Katushka/mKate from *Entacmaea quadricolor*.

THE HISTORY OF FLUORESCENT PROTEINS (CONT'D)

With his discoveries, another part of the fluorescent protein puzzle fell into place, as they, taken alongside the insights provided by the studies in Tsien lab, covered a portion of the color spectrum that GFP and its derivatives did not previously reach.

Nobel Prize for Fluorescent Proteins

The importance of fluorescent proteins was recognized in 2008 when the Nobel Prize in chemistry was awarded to Shimomura, Chalfie, and Tsien for their work on GFP. This great honor serves to highlight how useful fluorescent proteins like GFP really are. In this compilation we'll take a closer look at how they're used, see some new techniques that take advantage of them, and give some tips on deciding which one is right for you. So, if you're interested in learning more about fluorescent proteins, I invite you to read on and learn much more!

GREEN FLUORESCENT PROTEIN (GFP)

By Marcy Patrick | May 15, 2014

Bioluminescence and fluorescence from proteins such as Green Fluorescent Protein (GFP) has likely existed in creatures such as jellyfish for millions of years; however, it took until the 1960s for scientists to begin to study GFP and deduce its biochemical properties. Now GFP and its fluorescent derivatives are a staple in the lab. GFP is used in research across a vast array of biological disciplines and scientists employ GFP for a wide number of functions, including: tagging genes to elucidate their expression or localization profiles, acting as a biosensor or cell marker, studying protein-protein interactions, visualizing promoter activity, and much more.

Read on to learn more about GFP, how scientists have evolved this versatile protein to suit their experimental needs, and some of the common applications in the lab.

Mice expressing GFP. Image source Moen et al., 2012.

Why Green Fluorescent Protein?

GFP is a ~27 kDa protein consisting of 238 amino acids derived from the crystal jellyfish *Aequorea victoria*. It has a fluorescent emission wavelength in the green portion of the visible spectrum (hence the name), which is due to a chromophore formed from a maturation reaction of three specific amino acids at the center of the protein (Ser65, Tyr66, and Gly67). When first discovered, one of the most surprising aspects of GFP was the fact that the chromophore forms spontaneously and without additional co-factors, substrates, or enzymatic activity – it only requires the presence of oxygen during maturation. This meant that the protein could be taken directly from *A. Victoria* and expressed in any organism while still maintaining fluorescence.

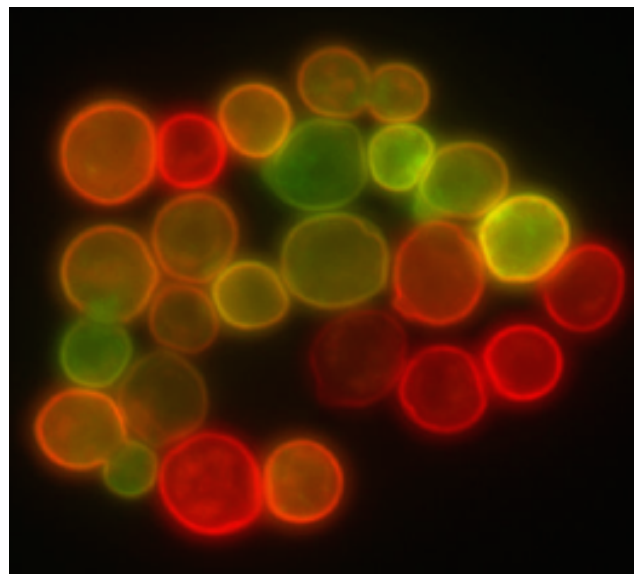
The protein structure, first reported in 1996, is an eleven β-sheet-containing “barrel” shape, with the chromophore concealed at the center of the structure, shielded from quenching by aqueous solvent. This tightly-packed structure explains the importance of the entire GFP protein, which is almost completely required to maintain fluorescent activity; very little truncation is tolerated, however, point mutations are acceptable. GFP’s main advantage over conventional fluorescent dyes of the time was the fact that it was non-toxic and could be expressed in living cells, enabling the study of dynamic, physiological processes.

Why Green Fluorescent Protein?

Almost as soon as its sequence was elucidated, scientists began engineering new versions of GFP through mutagenesis in order to improve its physical and biochemical properties. In 1995, Roger Y. Tsien described an S65T point mutation that increased the fluorescence intensity and photostability of GFP. This also shifted its major excitation peak from 395 nm to 488 nm, effectively ameliorating the deficiencies found in the wildtype protein and facilitating its widespread use in research. Many other mutations have since been introduced to GFP and new iterations of fluorophores are constantly being engineered. Table 1 below lists a few common fluorescent proteins and their mutations relative to wildtype GFP. Although not listed here, many permeations within each color also exist with only slight variations separating them.

GREEN FLUORESCENT PROTEIN (GFP) (CONT'D)

Please note that many fluorescent proteins found on the red side of the spectrum are not GFP derivatives, but are instead related to the dsRed protein isolated from *Discosoma sp*. Similar work has been done to expand the red-fluorescent protein repertoire; however, these proteins are unique from GFP and the mutation definitions found in Table 2 may not apply.



Yeast membrane proteins expressing GFP and RFP. Image source: Wikimedia Commons, accessed: 5/15/2014. Photo author: Masur.

A Multitude of Applications:

Due to its size and ease of use, GFP and other fluorescent proteins have become a mainstay in molecular biology. Scientists can easily utilize GFP-containing plasmids as a means to many functional ends. We've listed our favorites below, but many other uses currently exist, and new GFP technology is constantly being developed!

- **Fusion tagging**: One of the most common uses, GFP can be fused to the N- or C-terminus of a protein, which allows a scientist to visualize when and where the gene is expressed. Click here to view Addgene's collection of empty backbones for constructing fluorescent fusions.

- **Transcription reporter**: Placing GFP under the control of a promoter of interest can be used to effectively monitor gene expression from that promoter in a given cell type. This type of transcription reporting was among the earliest uses of GFP.

- **Förster resonance energy transfer (FRET)**: This is used to study the interactions between two proteins or between two domains of a protein that undergo conformational change. Typcially two fluorescent proteins with overlapping excitation/emission spectra are used; one fused to each protein or domain being tested. Find FRET plasmids here.

- **Split EGFP**: An alternative to FRET, split EGFP has also been used to study protein-protein interactions. In this case, two portions of EGFP are fused to the proteins of interest, and when they come into close proximity, the two halves of EGFP undergo folding, maturation, and fluorescence.

- **Biosensors**: A wide array of GFP-based fluorescent biosensors has been designed to detect a variety of intracellular conditions, including ion (such as Ca2+) concentrations and pH, using a range of strategies such as FRET, calmodulin, and others. Review Addgene's collection of fluorescent biosensors here.

- **Optogenetics**: Scientists can use light to detect, measure, and control molecular signals, cells, and groups of cells in order to understand their activity and visualize the effects of alterations to this activity. Learn more about optogenetics at OpenOptogenetics and find optogeneic actuators and sensors at Addgene.

- **Cell marking/selection**: Expression constructs like plasmids often include GFP as a marker to help identify which cells have successfully taken up the plasmid. This can serve as an alternative to antibiotic

GREEN FLUORESCENT PROTEIN (GFP) (CONT'D)

selection. Plasmids of this type may have the GFP under the control of an additional promoter from that of the gene of interest, or expressed from the same transcript as the gene of interest, but after an internal ribosome entry site (IRES). This is oftentimes used in conjuction with FACS (see below).

- **Fluorescence-activated cell sorting (FACS)**: This is a type of flow cytometry that separates mixtures of cells into distinct populations based on fluorescent signal. Thus, FACS can be used to separate cells expressing GFP from cells that are not.

- **Developmental/transgenic uses**: Because of its stability, GFP can be used in lineage tracking capacities in cell fate studies. It can also be used, when put under control of promoters of interest, to visualize the developmental stage at which these promoters are active. Further, GFP can label transgenically modified embryonic stem cells, which can then be used for implantation and generation of transgenic mice.

- **Purification**: GFP can be used as a general epitope tag for protein purification and a number of commercial antibodies to GFP are available.

- **Others**: We've really just scratched the surface of the potential applications for GFP. It has also been used to identify particular cell populations in drug screens, to visualize micrometastases in nude mice in cancer studies, act as a reporter for DNA double strand break repair, and to label pathogenic intracellular microbes to visualize host/pathogen interactions.

Table 1: The Specific Mutations Comprising Common Fluorophores

Fluorescent Protein	Mutations Relative to Wildtype GFP
EGFP	F64L; S65T
EYFP	S65G; V68L; S72A; T203Y
mYFP	S65G; V68L; Q69K; S72A; T203Y; A206K
Citrine	S65G; V68L; Q69K; S72A; T203Y
ECFP	F64L; S65T, Y66W; N149I; M153T; V163A
mCFP	F64L; S65T, Y66W; N149I; M153T; V163A; A206K
Cerulean	F64L, S65T, Y66W, S72A, Y145A, H148D, N149I, M153T, V163A
EBFP	F64L, S65T, Y66H, Y145F

GREEN FLUORESCENT PROTEIN (GFP) (CONT'D)

Table 2: Functional Role of Specific Mutations in GFP Derivatives

Mutation	Known Function
S65T	Increased fluoresence, photostability, and a shift of the major excitation peak to 488 nm
F64L	Increased folding efficiency at 37C
Y66W	Causes the chromophore to form an indole rather than phenol component (cyan derivatives)
Y66H	Blue-shifts the wavelength (blue derivatives)
Y145F	Increases quantum yield for BFP
Y145A and H148D	Stabilizes the structure of Cerulean derivatives
F99S, M153T, V163A	Improves folding at 37C, reduces aggregation at high concentrations
T203Y	Red-shifts the wavelength (yellow derivatives)
A206K	Interferes with dimer interface (monomeric derivatives)
K26R, Q80R, N146H, H231L, (and probably others)	Neutral mutations

WHICH FLUORESCENT PROTEIN SHOULD I USE?

By Gal Haimovich | May 20, 2014

Be honest. Do you really know how fluorescent proteins glow?

Fluorescent Proteins (FPs) were first discovered over 50 years ago, with the identification of the Green Fluorescent Protein (GFP), a protein from the jellyfish *Aequorea victoria*. Since that discovery, the family of FPs just keeps getting larger with hundreds of variants available. Read on to familiarize yourself with the available FP emission colors and 10 points to keep in mind when choosing an FP (or two) for your upcoming experiments.

Fluorescence is the emission of light by a substance that has absorbed light. The emitted light is at a longer wavelength than the exciting wavelength. Thus, FPs are proteins with this unique capacity.

Many of these FPs are fluorescent when ectopically expressed in most organisms. Furthermore, fusing an FP to another protein usually does not affect its fluorescence. Therefore, FPs are used to study many biological questions. The two most common uses are: 1) to test the expression level in a specific system (by measuring the fluorescence intensity); and 2) to visualize the localization of the FP (fused to the protein of interest), thus tracking the localization of that biomolecule inside living cells.

Glofish expressing a variety of fluorescent proteins. Source: glowfish.com

FPs Classified By their Emission Color (Emission Wavelength Range)

FP Color	Emission Wavelength
Blue	424 - 467 nm
Cyan	474 - 492 nm
Green	499 - 519 nm
Yellow	524 - 538 nm
Orange	559 - 572 nm
Red	574 - 610 nm
Far-Red	625 - 659 nm
Infra-Red	≥ 670 nm

FPs are usually classified by emission color as outlined to the left (or emission wavelenght range). By mutating GFP, the variants blue FP (BFP), cyan FP (CFP), and yellow FP (YFP) were derived. For a breakdown of GFP, it's variants, and their relevant mutations, check out the "GFP" section. Additionally, many other FPs have been found in other organisms.

WHICH FLUORESCENT PROTEIN SHOULD I USE? (CONT'D)

Unique Categories of FPs

In addition to emission wavelength range, there are other traits that need to be considered when choosing an FP:

- Photoactivatable / Photoconvertible: These proteins can switch their color when activated by a specific excitation wavelength. This means that the emission wavelength can change. In a few cases, the initial state of the protein is non-fluorescent, thus allowing a very low background level of fluorescence. Examples for such photoactivatable or photoconvertible proteins are PA-GFP, Dendra2, and the mEOS proteins. Some proteins are reversibly switchable (e.g. rsEGFP, Dreiklang).

- Fluorescent Timers (FT): These proteins change their color over time. Therefore, these can be used as "timers" for cellular processes following their activation. The four main FTs are called Slow-FT, Medium-FT, Fast-FT, and mK-GO.

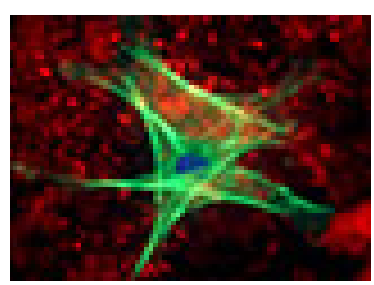
- Large Stokes Shift (LSS): Stokes shift (named after George G. Stokes) is the shift in wavelength from excitation to emission. For most FPs, Stokes shift is less than 50 nm (often much less). For LSS proteins, the Stokes shift is ≥ 100 nm. Specifically, these proteins are excited by UV light or blue light and they emit green or red light. For example, T-Sapphire, LSSmOrange, and LSSmKate.

- Fluorescent Sensors: These FPs change their excitation/emission behavior upon environmental changes (e.g. pH, Ca2+ flux, etc). The most commonly used are GECIs - genetically encoded calcium indicators (e.g. GCaMP). Others include: pHluorin & pHTomato (pH sensors), HyPer (H2O2 sensor), ArcLight (voltage sensor), and iGluSnFr (glutamate sensor). More examples of these biosensors can be found at Addgene.

- Split FPs: some FPs (e.g. GFP, Venus) can be split into two halves, which are non-fluorescent on their own. If the two halves are in close proximity, they will form the full FP and fluoresce. Split FPs can be used to determine the proximity of two proteins fused to the halves of the split FP. This technique is also is also called Bimolecular Fluorescence Complementation (BiFC).

8 Points to Keep in Mind When Choosing an FP

1. Excitation & Emission (ex/em):



Each FP has its own unique ex/em peaks. Therefore, choose FPs that your system can excite, and detect the emission. For example, if your microscope has only two lasers, at 488 nm and 561 nm, you will not be able to use far red-FPs. If you do not have a filter that will pass blue light to the detector/camera, then BFPs are of no use to you.

When using more than one FP, make sure their emission light does not overlap in wavelength. In many microscopes the filters are not narrow enough to distinguish between closely related colors. Furthermore, most FPs have a broad range of emission which will be detected by longer-wavelength filters (e.g. GFP also emits yellow light).

Note that some combinations of FPs can cause an effect called FRET (fluorescence [or Förster] resonance energy transfer). FRET occurs when energy transfer from one FP (e.g. CFP) excites the

WHICH FLUORESCENT PROTEIN SHOULD I USE? (CONT'D)

fluorescence of another FP (e.g. YFP). FRET only occurs when the distance between the two FPs is <10 nm, and should be considered when labeling proteins that interact. Indeed, FRET is often used to determine if two proteins interact.

2. Oligomerization

The first generations of FPs were prone to oligomerize. This may affect the biological function of the FP-fusion protein. Therefore, it is recommended to use monomeric FPs (usually denoted by a "m" as the first letter in the protein name, e.g. mCherry).

3. Oxygen

The maturation of the chromophore on many FPs (particularly those derived from GFP) requires oxygen. Therefore, these FPs cannot be used in oxygen deprived environment. Recently, a new GFP isolated from the Unagi eel was shown to mature independently of oxygen, making it suitble for use in anaerobic conditions.

4. Maturation Time

Maturation time is the time it takes the FP to correctly fold and create the chromophore. This can be from a few minutes after it is translated to a few hours. For example, superfolder GFP (sfGFP) and mNeonGFP can fold in <10 min at 37 °C, mCherry takes ~15 min, TagRFP ~100 min and DsRed ~10 hours.

5. Temperature

FP maturation times and fluorescent intensity can be affected by the temperature. For instance, enhanced GFP (EGFP) was optimized for 37 °C, and is therefore most suited for mammalian or bacteria studies, whereas GFPS65T is better suited for yeast studies (24-30 °C).

6. Brightness

Brightness is calculated as the product of extinction coefficient and quantum yield of the protein, divided by 1000. In many cases the brightness is compared to that of EGFP which is set as 1. Some proteins are very dim (e.g. TagRFP657, which has a brightness of 0.1) and this should be taken into account.

7. Photostability

Fluorescent molecules gets bleached (i.e. lose the ability to emit light) after prolonged exposure to excitation light. Photostability can be as short as 100 ms (EBFP) or as long as 1 hour (mAmetrine1.2). However, for most FPs it is a few seconds to a few minutes. Photostability can be affected by experimental parameters (e.g. excitation light intensity, pH, or temperature).

8. pH Stability

This parameter is important if you are planning to express the FP in acidic environments (e.g. yeast cytosol, which is slightly acidic, or synaptic vesicles). Some FPs have different ex/em spectra (e.g. mKeima) or change fluorescent intensity upon pH changes (e.g. pHluorin, pHTomato).

WHICH FLUORESCENT PROTEIN SHOULD I USE? (CONT'D)

Keep this list handy as you plan your next experiment or to hand to the next labmate who asks you, "Which fluorescent protein should I use?" And if you're looking for more fluorescence microscopy tools and techniques to aid your work, head over to greenfluorescentblog.

Helpful Websites and FP Resources

More Helpful Websites & Resources

- Addgene's Fluorescent Protein Guide Pages
- Fluorescence Spectrum Viewer from BD Bioscience
- Interactive Visualization of Fluorescent Protein Properties
- Fluorescence SpectraViewer from Invitrogen (Life Techonologies)
- ilovegfp - site with very comprehensive datasheets on many FP variants

Further Reading

1. Stepanenko, Olesya V., et al. "Fluorescent proteins as biomarkers and biosensors: throwing color lights on molecular and cellular processes." Current Protein and Peptide Science 9.4 (2008): 338-369. PubMed PMID: 18691124. PubMed Central PMCID: PMC2904242.

2. Chudakov, Dmitriy M., et al. "Fluorescent proteins and their applications in imaging living cells and tissues." Physiological reviews 90.3 (2010): 1103-1163. PubMed PMID: 20664080.

3. Wu, Bin, et al. "Modern fluorescent proteins and imaging technologies to study gene expression, nuclear localization, and dynamics." Current opinion in cell biology 23.3 (2011): 310-317. PubMed PMID: 21242078. PubMed Central PMCID: PMC3143818.

CHOOSING YOUR FLUORESCENT PROTEINS FOR MULTI-COLOR IMAGING

By Kurt Thorn | October, 2014

A common requirement for live cell imaging experiments is the ability to follow multiple fluorescently tagged species simultaneously. To do so with fluorescent protein labels requires multiple fluorescent proteins whose excitation and emission spectra differ sufficiently for them to be imaged in distinct fluorescent channels on the microscope. With the proliferation of fluorescent proteins in recent years, there are many fluorescent protein combinations that can be imaged together, but this also means that the choice of fluorescent proteins requires some thought.

The first step in choosing fluorescent proteins for your multi-color imaging experiment is to be aware of what fluorescent proteins are available. With new fluorescent proteins being published every month, deciding on the best protein for a given application is a challenge. To help keep you abreast of the latest fluorescent proteins, I maintain an interactive graph and table of the best fluorescent proteins currently available.

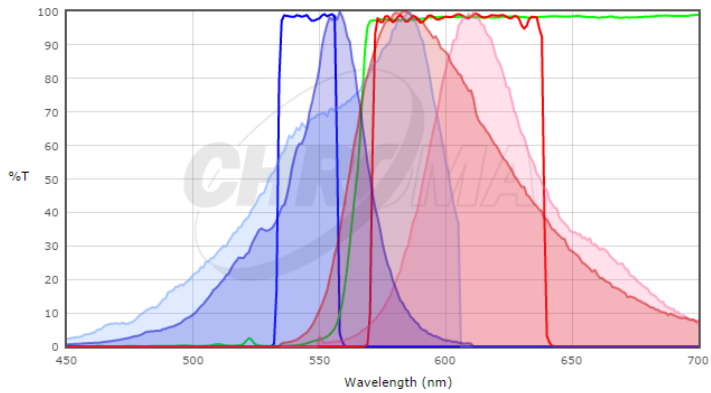
Choosing Compatible Fluorescent Proteins

To choose a set of fluorescent proteins to be imaged together, you will need to consider the same factors as when choosing an individual fluorescent protein (brightness, photostability, and so on; see "Which Fluorescent Protein Should I Use?"). In addition, you will also need to choose fluorescent proteins that can be distinguished from one another and that can be imaged with the optics on the microscope(s) you intend to use. An accurate determination of whether two fluorescent proteins can be separated from each other requires knowledge of their excitation and emission spectra, but a good rule of thumb is that both the peak excitation wavelengths and peak emission wavelength of the two proteins should be separated by 50-60 nm. For example, CFP (ex 430 nm / em 474 nm) and YFP (ex 514 nm / em 527 nm) can be imaged together but CFP and GFP (ex 488 nm / em 507 nm) show some crosstalk between the two fluorescent proteins. If you must image fluorescent proteins whose spectra overlap, there are techniques, like spectral unmixing, which can be used to separate the fluorescent proteins, but these are beyond the scope of this eBook.

Are Your Fluorescent Proteins Compatible with Your Microscope Optics?

To determine if the fluorescent proteins you are interested in are compatible with your microscope optics, you will want to compare the excitation and emission spectra of your protein with the filter sets or lasers on your microscope. Ideally, you would like to have substantial overlap between the excitation and emission filters and the excitation and emission spectra of the protein, so that the protein is well excited by your microscope and the fluorescence emission of the protein is efficiently collected by the microscope. To compare the match between a fluorescent protein and a filter set, many filter set vendors provide tools to plot the fluorescence spectra of proteins and dyes and their filters (see Chroma's, Semrock's, or Omega's). While these don't contain all fluorescent proteins in common use (particularly not the most recently published ones), they can be a good starting point. In many cases it is sufficient to use a spectrum for a closely related protein, if you know that your protein of interest has a similar spectrum. For example, one the next page, you'll find a screenshot from the Chroma Spectra Viewer comparing a standard Cy3 or Rhodamine filter set (Chroma #49004) to the spectra of both mCherry and TagRFP.

CHOOSING YOUR FLUORESCENT PROTEINS FOR MULTI-COLOR IMAGING (CONT'D)



Here, the TagRFP spectrum is shown in the darker colors and the mCherry spectrum is shown in the lighter colors; excitation spectra are blue and emission spectra are red. Neither is a perfect match to the filter set but the excitation filter excites more of the peak of the TagRFP excitation and the emission filter collects a larger fraction of the TagRFP emission than the mCherry emission. For this filter set, we would expect TagRFP to give a brighter signal than mCherry. In general, filter sets designed for Rhodamine / Cy3 will work better with shorter wavelength red fluorescent proteins like TagRFP or mRuby2 than longer wavelength proteins like mCherry. For background on fluorescence and filter sets, see the Introduction to Fluorescence Microscopy lecture at iBiology.

Commonly Used Filter Sets & Relevant Fluorescent Proteins

Commonly used filter sets for multicolor imaging include ones designed for CFP, YFP, and RFP or the Sedat Quad filter set, designed for DAPI / Fluorescein / Rhodamine / Cy5 (e.g. Semrock's) and the similar 4-laser combination on a confocal (405 / 488 / 561 / 640 nm). In our hands the best fluorescent proteins for imaging with this set are mTagBFP2, EGFP or one of the improved GFP variants, mRuby2 or TagRFP-T, and an infrared fluorescent protein such as iFP1.4 or iFP2.0. Beware that these infrared fluorescent proteins require biliverdin as a cofactor and so you may need to supplement your cells with biliverdin for maximal brightness. In mammalian cells, one of the improved folding variants of EGFP like mEmerald or Clover is probably best; mNeonGreen is an even newer green fluorescent protein that is supposed to be extremely bright. In *S. cerevisiae*, we've tested a number of green and red fluorescent proteins with this filter set and have reported brightness measurements. Here, EGFP outperforms the improved folding variants, presumably due to the lower growth temperature. This also suggests, however, that there is no single fluorescent protein optimal for all organisms and that if you want the brightest signal, you may need to try several proteins in your system of interest. Finally, in this set of proteins the green and red proteins are generally the most detectable and so should be used to tag your least abundant proteins, with the blue and infrared channels used for more abundant proteins or marking compartments.

I hope this sheds some light on multicolor imaging with fluorescent proteins. With the right microscope and the right choice of fluorescent proteins, imaging four colors simultaneously should be pretty straightforward.

Further Reading

1. Check out the Thorn Lab Web Page
2. Read Kurt's Microscopy Blog

A PRACTICAL APPROACH TO CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN

By Joachim Goedhart & Marieke Mastop | August 26, 2015

Before you decide which car you want to buy, it is worthwhile to test-drive a couple of candidates. Before you buy a new microscope, it is smart to look at (and through) a couple of models. Before you start a new project with fluorescent proteins, the best advice is to try a couple of promising variants to check how they perform under your experimental conditions. This is time well spent and, if you do it right, can be (part of) figure 1 of your next paper or thesis. This series of posts explains how to critically assess the reported properties of fluorescent proteins, how to do a head-to-head comparison of fluorescent proteins and how to make a well-informed decision on the best fluorescent protein for your application.

Selecting the best fluorescent protein for a specific application can seem like a daunting task. Many fluorescent proteins are available and the number of fluorescent proteins is steadily increasing. Often, the choice for a fluorescent protein is based on the properties found in the literature or tabulated data that summarizes properties of fluorescent proteins on websites. To make the best choice, the fluorescent protein properties need to be scrutinized under relevant conditions.

The properties of fluorescent proteins are usually only examined under a few conditions since it is impossible to check all possible combinations of biology and equipment. Because the performance of a fluorescent protein strongly depends on both the biological system as well as the imaging method, we recommend basing your selection on a small-scale head-to-head comparative study under conditions that best mimic the intended application. The three key properties for live cell imaging that will be treated in this and the next two sections (i) brightness, (ii) photostability and (iii) aggregation tendency.

Brightness

Brightness refers to the fluorescence intensity of a fluorescent molecule. A higher brightness increases the detected signal and therefore high brightness is an important feature of a fluorophore. The key question is how the brightness is best defined or determined. Here, we will explain how practical brightness is determined and why practical brightness is a better criterion than theoretical brightness for selecting the brightest fluorescent protein.

Theoretical Brightness

Two key properties of fluorophores that determine brightness are the extent to which the excitation light is absorbed and the efficiency by which absorbed photons are converted into emitted photons. These are indicated by the extinction coefficient (EC) and quantum yield (QY) respectively. The theoretical brightness is calculated by multiplying EC by QY (EC*QY, sometimes normalized to the value of EGFP). The higher the number, the higher the theoretical brightness. The theoretical brightness often appears in tables with fluorescent protein properties and provides a quick way to compare fluorescent proteins, e.g. see Chudakov et al (2010), Cranfill et al (2016), or Thorn (2017). However, the theoretical brightness ignores several important experimental conditions that are related to the imaging strategy and the sample.

Practical Brightness

A protein with a high intrinsic brightness that does not fold well or cannot be detected in a microscopy setup has zero practical brightness and is of no use. Therefore, it is more sensible to compare fluorescent proteins based on their practical brightness in your experimental set up. The practical brightness takes all application specific parameters into account, including the specs of your microscope (excitation wavelength, available

A PRACTICAL APPROACH TO CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN (CONT'D)

emission filters, and detector sensitivity) and the biological system (temperature, prokaryote versus eukaryote, background fluorescence).

Determining Practical Brightness

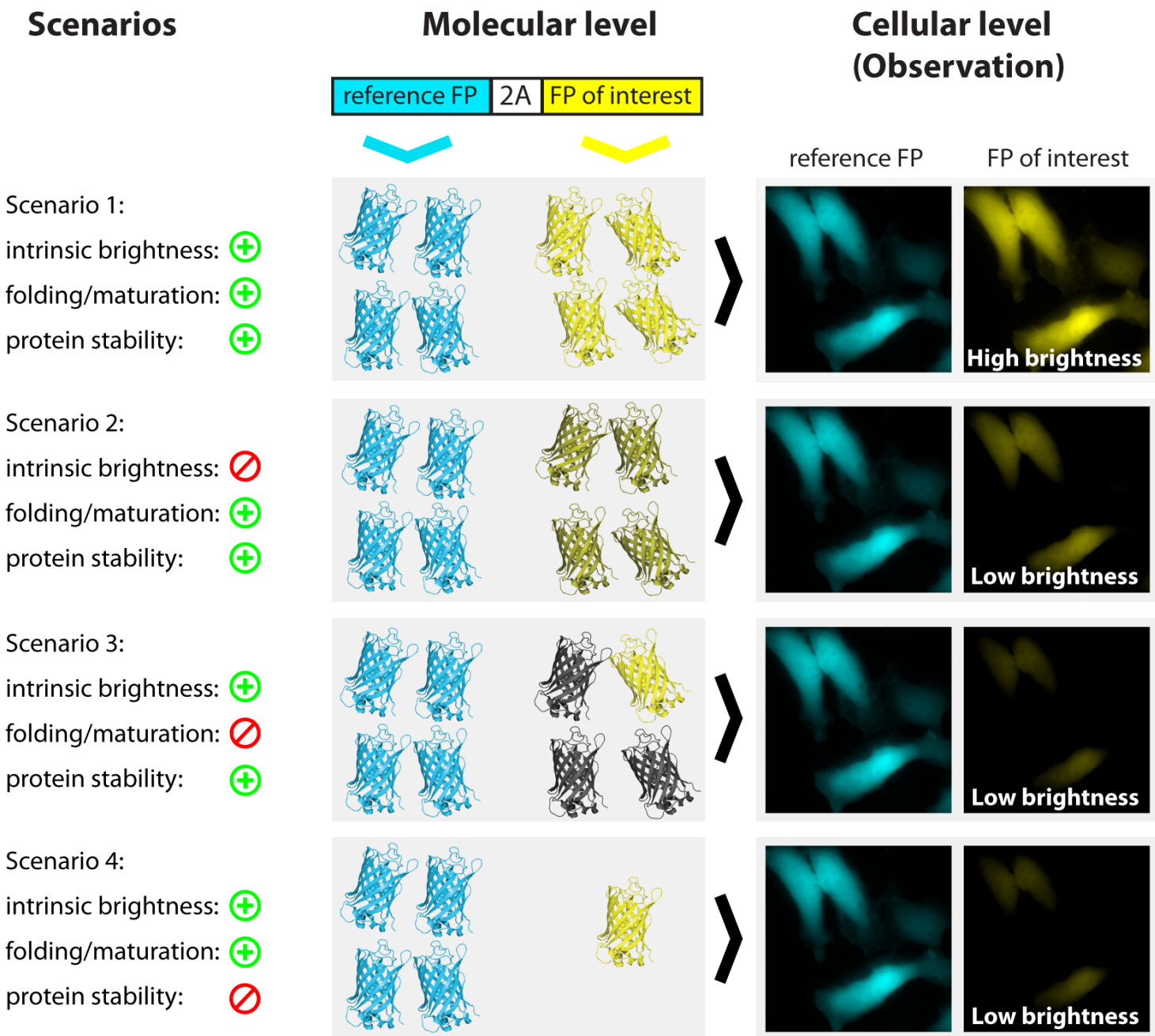


Figure 1. An illustration of a cell-based assay used to determine practical brightness. A reference fluorescent protein (FP) is co-expressed together with the FP of interest in an equal amount from a single plasmid. The reference FP is used to account for cell-to-cell variation in transfected cells. Bright cells will be observed when the protein has high intrinsic brightness, folds well, and has good protein stability (scenario 1). The practical brightness will be lower when the intrinsic brightness of the individual FP is low (scenario 2), a part of the protein does not mature (scenario 3) or when the fluorescent protein is rapidly degraded (scenario 4). Suboptimal imaging settings will also result in a low practical brightness.

A PRACTICAL APPROACH TO CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN (CONT'D)

When a dish of mammalian cells is transiently transfected with a plasmid containing a fluorescent protein (fusion) there is huge variation in the fluorescence intensities of individual mammalian cells within the dish. This is caused by the stochastic nature of plasmid uptake by cells. To correct for this variation, a reference fluorescent protein that is expressed at an equal level (Goedhart et al, 2011) can be used.

An assay developed in our lab uses a reference protein that is translated from the same open-reading frame and separated from the protein of interest by a 2A self-cleaving peptide (Goedhart et al, 2010). An explanation of the assay is depicted in Figure 1. The strict correlation between the fluorescent protein of interest and the reference protein (Goedhart et al, 2011) allows for a precise determination of brightness in cells, under realistic experimental conditions. By determining the brightness for a set of fluorescent proteins (with the same reference protein) under identical conditions, a ranking of the practical brightness can be made (Goedhart et al, 2012, Bindels et al, 2017). The plasmid for co-expressing mTurquoise2 with mVenus as a reference protein is available from Addgene.

Another way to dodge cellular variation observed in mammalian cells is to tag an endogenous gene. By imaging cells (or tissues) that produce an endogenous protein tagged with a fluorescent protein and repeating this with another fluorescent protein, a ranking of practical brightness can be made. This strategy has been used in yeast by Lee et al (2013) and in nematodes by El Mouridi et al (2017) and Heppert et al (2016). In mammalian cells this would be possible with CRISPR/Cas-based genome editing.

Selecting a Bright Fluorescent Protein

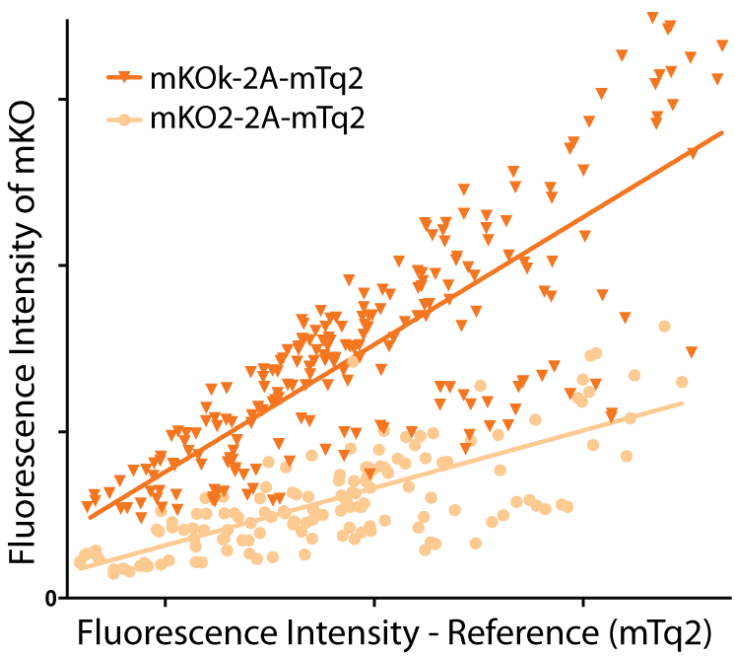


Figure 2. Experimental data obtained from the practical brightness assay performed on two orange fluorescent proteins. The orange fluorescence emitted by mKO2 or mKOk is plotted against the emission from the reference protein. Each dot represents the intensity of a single cell. The data shows a 2-fold higher practical brightness for mKOk relative to mKO2. This is striking since the difference between the FPs is only one amino acid: mKOk = mKO2(M176F).

Practical brightness takes all experimental conditions into account. In particular, the folding and maturation efficiency of the fluorescent protein in the host cell, which is a key determinant of the practical brightness. Therefore, practical brightness provides a better picture of what can be expected in a 'real' application than the theoretical brightness. Consequently, it makes sense to identify a couple of promising fluorescent proteins and to determine and compare their practical brightness in a system closely mimicking the future application. In Figure 2 we provide an example of the comparison of the practical brightness of two orange fluorescent proteins, differing by only a single amino acid. Without further information about properties like photostability or aggregation tendency, our choice would be mKOk; it is much brighter according to this data. The practical brightness assay (employing co-expressing fluorescent proteins or using endogenous tagged genes) can be used to verify performance under different imaging conditions (e.g. different emission filters) or different set-ups.

CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN: PHOTOSTABILITY

By Joachim Goedhart & Marieke Mastop | May 30th, 2017

Photobleaching is the irreversible destruction of a fluorophore under the influence of light. Any fluorescent molecule will photobleach at some point. For live-cell imaging, it is desirable to have fluorescent proteins that are photostable. On top of photobleaching, fluorescent proteins may display reversible intensity changes (Shaner et al, 2008; Bindels et al, 2017) and photoswitching (Kremers et al, 2009), which usually are undesired properties. In the ideal situation, a fluorescent protein should emit a stable fluorescence signal, showing no or little deterioration or change of the signal during the course of the experiment.

The best fluorescent proteins for live cell imaging can be excited many times, thereby producing a large number of emitted photons before they are destroyed.

Factors that Affect Photostability

The photobleaching rate of a fluorophore primarily depends on the excitation power and the excitation wavelength. A fluorophore is less likely to bleach when it is excited off the peak wavelength, since it will absorb the excitation light less efficiently. Similarly, decreasing the power of the excitation light reduces the number of excitation/emission cycles per unit of time, decreasing the likelihood that a fluorophore will bleach. This is, however, not the full story. The photobleaching rate does not depend on excitation power in a linear fashion. This implies that reducing the power 2-fold does not reduce photobleaching by half. How exactly the photobleaching rate depends on power is a property of the fluorescent protein. This can differ between fluorescent proteins in the same spectral class (Cranfill et al, 2016).

This non-linear dependence on excitation power is important since different fluorescence imaging strategies use widely varying amounts of excitation power (Shaner et al, 2008). In confocal laser scanning microscopy, fluorescent proteins are excited with very intense light (for a brief time), whereas in widefield imaging fluorophores are excited with relatively low light levels (for a prolonged time). Other illumination strategies, such as 2-photon-excitation, selective plane illumination, TIRF or spinning disk confocal use entirely different excitation power regimes. Therefore, it is unpredictable which fluorescent protein is most photostable for each of these conditions. On top of these factors, environmental conditions such as cellular redox state and oxygen concentration may affect photobleaching rates (Shaner et al, 2008). This brings us to the key question: what is the best way to compare the photostability of different fluorescent proteins?

Measuring Photostability

Since it is clear that a photostable fluorescent protein is a key requirement for quantitative live-cell imaging, it is important to be able to quantify the photostability of fluorescent proteins. To determine photostability, an experiment is performed that measures fluorescence intensity over time. In order to predict how fluorophores perform in 'real experiments', it is advisable to perform time-lapse imaging of cells producing the fluorescent protein of interest under realistic conditions, i.e. with low excitation power. By repeating these measurements for different fluorescent proteins and by comparing how the fluorescence intensity changes over time, the photostability of the fluorescent proteins can be directly compared. An example of a photostability measurement of various cyan fluorescent proteins is depicted in Figure 1. Of note, the photobleaching rate does not depend on fluorescence intensity or protein distribution. Therefore, photostability measurements can be performed with soluble fluorescent proteins or localized fusion proteins and do not require dedicated plasmids or constructs.

It should be noted that the photostability measurements reported in the literature are performed in different

CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN: PHOTOSTABILITY (CONT'D)

ways. We describe a number of issues with some of the experimental designs used to make these measurements and hope this information can help guide your assessment of reported data.

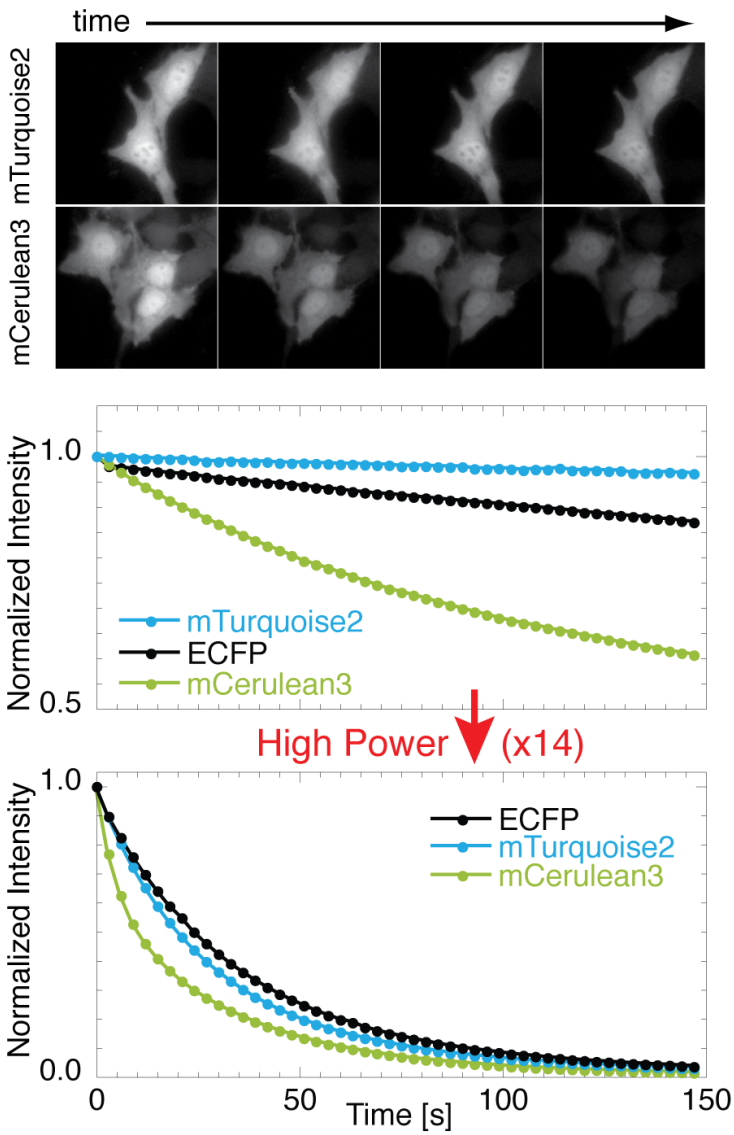


Figure 1. A comparison of the photostability of different cyan fluorescent protein variants expressed in HeLa cells. HeLa cells are continuously illuminated (widefield) at low excitation power and the fluorescence intensity is monitored by time-lapse imaging. Quantification of the signal from individual cells reveals how fluorescence evolves over time. Performing the same experiment at 14-fold higher excitation power shows that ECFP is less sensitive than mTurquoise2 to the increase power. The graphs are based on data that is reported by Goedhart et al, 2012.

Photobleaching results in a loss of fluorescence, which, in its simplest form, can be described by a mono-exponential decay (analogous to radioactive decay). Consequently, the intensity of decay is often described by the t½, which is the time after which half of the initial fluorescence intensity remains. The first issue is that, in several cases, the fluorescence intensity decay of a fluorophore does not follow a simple mono-exponential decay (Shaner et al, 2008; Bindels et al, 2017) and cannot be described by a single parameter. Therefore, it is necessary to know how the fluorescence intensity evolves over time.

The second issue is that, in experiments that measure photostability, high excitation powers are often used to reduce the length of the experiments. As the photobleaching rate is not linearly related to power, the conclusions reached at high power may not translate to real applications in which far less power is used. For example you might find that you can get useful data from your chosen FP for much longer than predicted by these high power experiments. Measuring photostability at realistic powers will provide better insight into the photostability in the intended application (Goedhart et al, 2012).

The third issue relates to the environment of the fluorescent protein. Photostability measurements can be performed on purified fluorescent proteins. To avoid diffusion, the proteins are (i) trapped in microdroplets in a water/oil emulsion, (ii) embedded in a gel, or (iii) attached to a substrate. These *in vitro* methods allow for a well-controlled environment, but they do not mimic the natural situation. Measuring photostability in living cells provides a much more realistic view of photostability.

Selecting a Photostable Protein

The issues with quantification of photobleaching rates can be summarised as follows: Instead of determining a t½ of a fluorescent protein in a water/oil emulsion at high power, it is far more relevant to measure how fluorescence intensity evolves over time in living cells at a power used for live-cell imaging.

To conclude, the most photostable fluorescent protein can be identified by a head-to-head comparison of

CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN: PHOTOSTABILITY (CONT'D)

several fluorescent proteins of the same spectral class on the microscopy system and in the cell type (or tissue) that is intended to be used with the fluorescent protein. In Figure 1 we provide an example of such a comparison between cyan fluorescent proteins using widefield imaging. Our choice for a photostable protein for live cell imaging would be mTurquoise2, based on the data acquired at low excitation powers. Note, however, that, at a 14-fold higher excitation power, the photostability of the variants is comparable (lower panel of figure 1).

To avoid changes in excitation power, it is important to perform the experiments on a single day on a stable system. A final piece of advice is to measure the excitation power you use in your imaging experiments. It is good practice to measure the excitation power (Grünwald et al, 2008) occasionally and certainly after a set-up has been changed (for instance after replacing a broken lightbulb or exchanging excitation filters). This avoids substantial changes in excitation power and will help to keep the photostability of the probes in check.

CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN: AGGREGATION TENDENCY

By Joachim Goedhart & Marieke Mastop | May 30, 2017

In the jellyfish *Aequorea victoria*, AvGFP forms a homodimer. In corals, the red fluorescent proteins form tetramers. In general, fluorescent proteins have a natural affinity and a tendency to form higher order aggregates. This property can be tolerated in some applications (e.g. labeling of cells or tracking promotor activity), but it is problematic in applications in which the fluorescent protein is used as an inert protein module. This is explained in more detail here. There are a variety of methods that can be used to measure your fluorescent protein's propensity to aggregate. The basics and pitfalls of these experiments are discussed here.

In Vitro Tests for Aggregation

The tendency of purified fluorescent proteins to form homodimers or higher order oligomers can be analyzed by several techniques. Gel filtration and SDS-PAGE under non-denaturing conditions separate proteins based on size. Whether higher molecular weight complexes are detected will depend on the homodimer affinity and the concentration of the protein in solution. During the experiment, the solution is diluted, complicating the interpretation. Because of this, these methods only give a qualitative view of dimerization tendency. Another technique that separates molecules based on size is ultracentrifugation. Sedimentation equilibrium analytical ultracentrifugation yields an affinity and has been used to demonstrate that yellow fluorescent protein homodimerization is characterized by an affinity of 110 μM (Zacharias et al, 2002), meaning that at this concentration, 50% of the protein forms dimers. Finally, spectroscopic methods (based on fluorescence polarization) can be used to detect homodimerization. However, all the aforementioned methods only determine the capacity of homodimerization in aqueous solution.

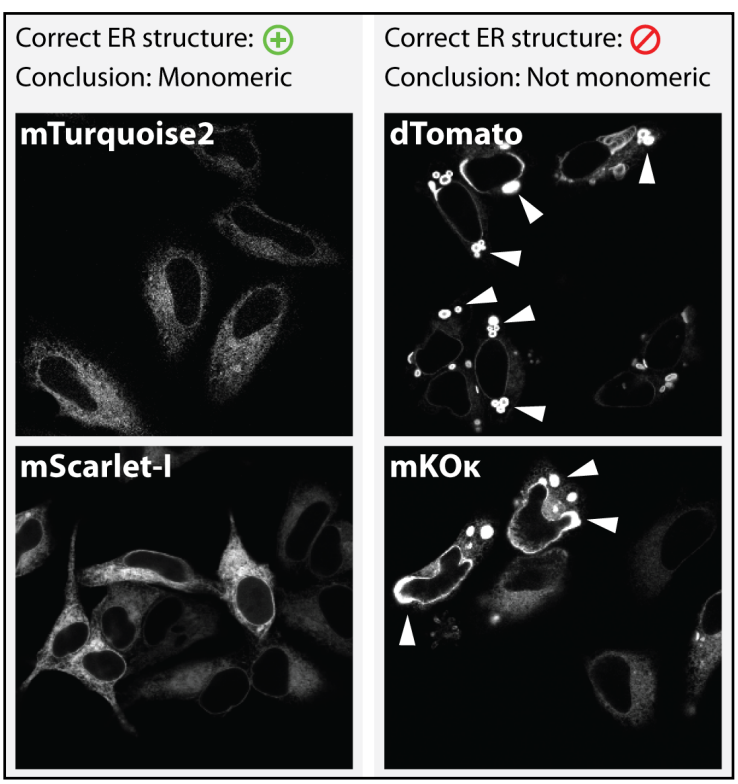


Figure 1. An OSER assay performed on HeLa cells with fluorescent proteins to assess dimerization tendency. A fusion of CytERM with the established monomeric FP, mTurquoise2, shows homogeneous endoplasmic reticulum (ER) labeling. In contrast, the dimerizing FP, dTomato, shows intensely labeled structures, known as OSER whorls (indicated with arrowheads). CytERM fusions with mScarlet-I show correct ER labeling and a similar fusion with mKOk shows numerous OSER whorls (indicated with arrowheads).

In Cyto Tests for Aggregation

To what extent does homodimerization assayed with purified protein, reflect the likelihood that fluorescent proteins interact or oligomerize in cells? This question is largely unanswered. Ideally, fluorescent proteins should act as inert modules that fail to homodimerize in cells. To understand how well fluorescent proteins approach this ideal situation, cellular assays have been proposed. Early efforts tested whether or not FP-tubulin fusion proteins could properly form microtubule networks in cells. Other efforts tested whether FP fusions to connexin localized correctly to gap junctions (Shaner et al, 2008). Obligate dimers (such as dTomato) that promote interactions between the FPs interfere with native interactions, preventing proper localization in these assays. However, these strategies will not detect weak dimerization tendency, as is present in EGFP.

Constantini et al, (2012) developed a cell-based assay that does a better job of detecting homodimerization tendency. This assay employs

CHOOSING THE B(RIGHT)EST FLUORESCENT PROTEIN: AGGREGATION TENDENCY (CONT'D)

a peptide derived from cytochrome p450, CytERM, to direct the fluorescent protein to the Endoplasmic Reticulum (ER). If homodimerization occurs (most likely in an antiparallel configuration), typical structures known as Organized Smooth Endoplasmic Reticulum (OSER) whorls are visible. These whorls can be quantified for individual cells, and the number of 'normal' looking cells can be counted. This assay will provide information on the dimerization tendency in living cells. Strikingly, the OSER assay reveals that tagRFP does not act as a true monomer in cells (Constantini, 2012), whereas it was previously concluded to be monomeric based on gel filtration (Chudakov et al, 2007).

When using and interpreting the results of the OSER assay, you should keep in mind that the presence of whorls is probably concentration dependent and cell-type dependent. Therefore, it is good practice to perform this assay with your chosen FP in your cell type of interest before starting your experiments.

Selecting a Non-oligomerizing Protein

A fluorescent protein cannot be assumed to be non-dimerizing if its name is preceded by an 'm' to declare that it is monomeric. The claim that a fluorescent protein is monomeric without the accompanying data to prove it is worthless. The OSER assay is currently the best way to test a fluorescent protein's propensity to homodimerize. Several studies have used the OSER assay to demonstrate monomeric behaviour in cells (Shaner et al, 2013; Bindels et al, 2017).

Still, the data presented in papers should be used with caution, since the difference between studies is substantial (e.g, the poor performance of mRuby2 is detected by Constantini et al (2015) and by Bindels et al (2017) but not by Cranfill et al (2016)). Moreover, good performance in the OSER assay is not a guarantee that using this fluorescent protein to tag your protein of interest will be problem-free.

In Figure 1 we provide an example of the OSER assay with a number of fluorescent proteins. Fusions of CytERM with mTurquoise2 and dTomato, which are established monomeric and dimerizing FPs respectively, are used as positive and negative controls for proper ER structure. Based on these data, we conclude that mKOk has dimerization tendency and is not suitable for protein tagging. On the other hand, mScarlet-I shows correct ER labeling, which is good evidence that mScarlet-I acts as a monomer in the cell type tested.

To conclude, we recommend that you try a couple of different fluorescent proteins when protein tagging is the goal (Cranfill et al, 2016; Constantini et al, 2012). The localization and biological properties of new fusions can be compared with mEGFP or mTurquoise2, which are established true monomeric fluorescent proteins.

Concluding Remarks

Many fluorescent proteins are available, and their number is steadily increasing. Of course, you want the 'latest and greatest' variants with the best properties for your research. But be aware, that the fluorescent proteins reported in publications are only characterized under a limited number of conditions, often using purified proteins. Therefore, it is essential to verify a number of key properties (brightness, photostability, oligomerization) of a number of promising fluorescent proteins under the conditions that are relevant for your research.

This will reveal the performance of fluorescent proteins in your biological system with the microscope that you will be using. If you do a head-to-head comparison right, it will be valuable information for the scientific community. The knowledge and experience that is gathered can be shared by publishing it as (part of) figure 1,

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uploading it as a preprint, or documenting it in some other way. Besides the data, new plasmids and/or cells or organisms that are engineered can be shared to simplify the comparison by others. Together, we can build a valuable resource with tools and data that indicate the performance of (a set of) fluorescent proteins under specific conditions.

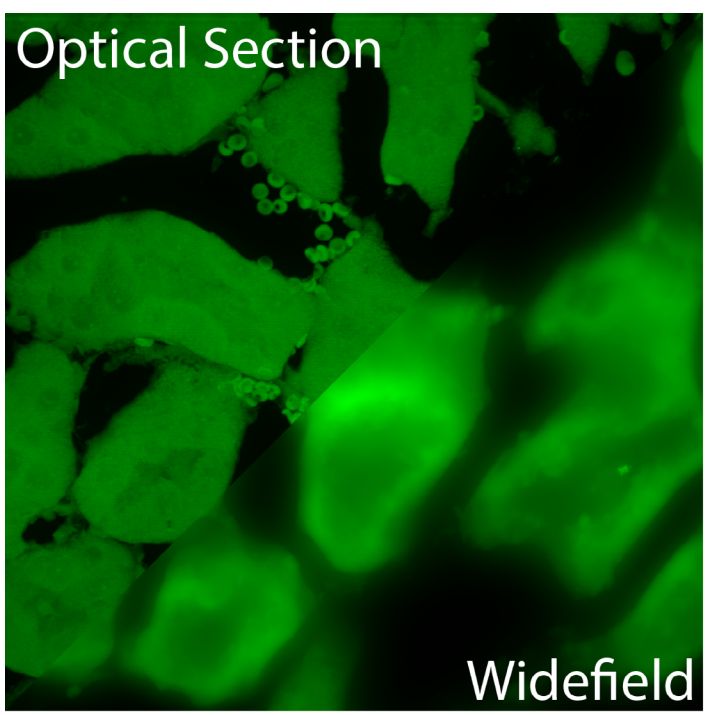
FLUORESCENCE MICROSCOPY TECHNIQUES

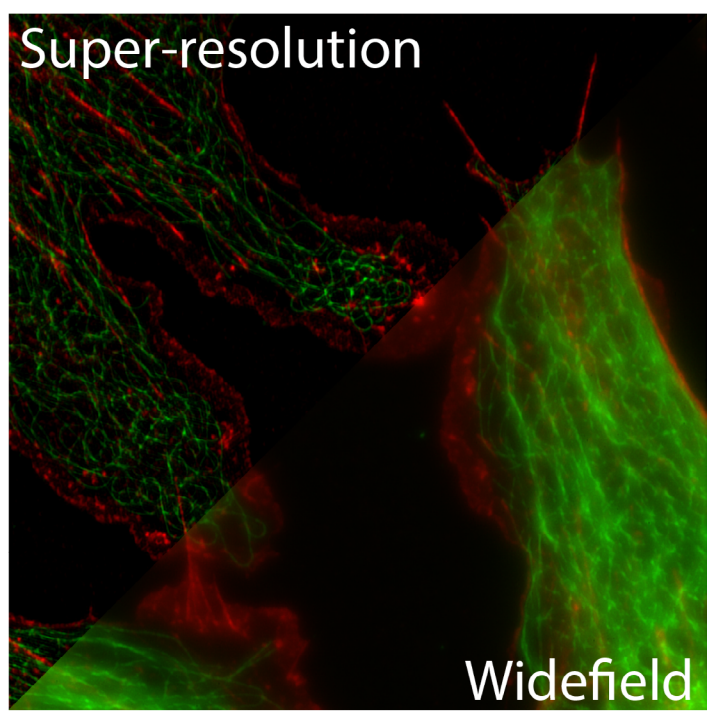
By Doug Richardson | June 22, 2017

No matter whether you are a sports photographer at the Super Bowl, a medical technologist taking an x-ray, or a biologist imaging the smallest structures of life; the key to a great image is contrast. The human visual system relies primarily on contrast to identify individual objects and perceive the world around us. Without contrast, objects simply vanish into noise.

Due to its unrivaled contrast, fluorescence imaging has emerged as the dominant light microscopy contrast technique in modern biology (1). When performed correctly, fluorescence microscopy provides a high contrast image in which a bright signal overlays a perfectly black background. In addition to this, the use of multiple fluorophores can add a second layer of contrast, color contrast, to an image and thereby provide molecular or structural specificity to the viewer. Finally, modern microscope designs may further exploit the unique characteristics of fluorophores to block out of focus fluorescence from reaching a detector or restricting fluorescence to specific excitation volumes to enhance spatial contrast (i.e. optical sectioning (2) or super-resolution (3).

Fluorescence Microscopy Techniques





Fluorescent Widefield Microscopy – is the most common and simplest form of fluorescent microscopy. Collimated (non-converging or diverging) excitation light exits the microscope objective evenly illuminating the entire (wide) field of view. Fluorescent light traveling back towards the objective is collected and focused onto a camera for visualization. Illuminating a sample in the opposite direction to fluorescence collection is referred to as “Epi” illumination. Therefore these are sometimes call Epi-fluorescence microscopes. (See left image above for an example image).

Point Scanning Confocal Microscopy – was the first fluorescence microscopy technique to incorporate optical sectioning. Optical sectioning refers to the ability to extract light from a single, thin plane within a relatively thicker three dimensional sample (see above, left); similar to an MRI or CT scanner. This is

FLUORESCENCE MICROSCOPY TECHNIQUES (CONT'D)

accomplished by focusing excitation light to a point in the sample and raster scanning the point to build up a final image pixel by pixel. The collected fluorescent light passes through a pinhole before reaching the detector. The pinhole is specifically placed to only allow light from the focal plane to reach the detector and all light from above or below is excluded.

Parallelized Confocal Microscopy (Spinning Disk) – increases the speed at which a confocal image can be acquired. Assembling an image pixel by pixel is slow; therefore, certain confocal microscopes use parallelization to enhance performance. In spinning disk microscopy, a metal disk containing a number of holes rotates through the excitation light path. Each hole corresponds to a different location in the sample. As more than one hole is illuminated at a time, the image can be acquired more quickly.

2-Photon Microscopy – attempts to solve two drawbacks of widefield and confocal microscopy. First, widefield and confocal microscopy project excitation light through the entire axial volume of a sample. Therefore, when a large number of optical slices are acquired all fluorophores throughout the sample are constantly exposed to light, not just those in the focal volume. This leads to more rapid photobleaching and a reduction in signal intensity. 2-Photon microscopes restrict excitation (and bleaching) to a single focal point. This is accomplished by using, for example, red light instead of blue to excite a molecule of GFP. Because red light has approximately half the energy of blue light, two photons of red light are needed to excite GFP compared to only one of blue. Only at the focal point of the objective can a a high enough density of red-photons be establish for this to happen.

Using red light has a second advantage: red light penetrates deeper into biological tissue. To prove this, simply hold a flash light to the palm of your hand. Although white light is entering your hand, only orange/red light can be seen exiting through the tissue. Therefore, 2-photon allows for deeper imaging into thick tissues.

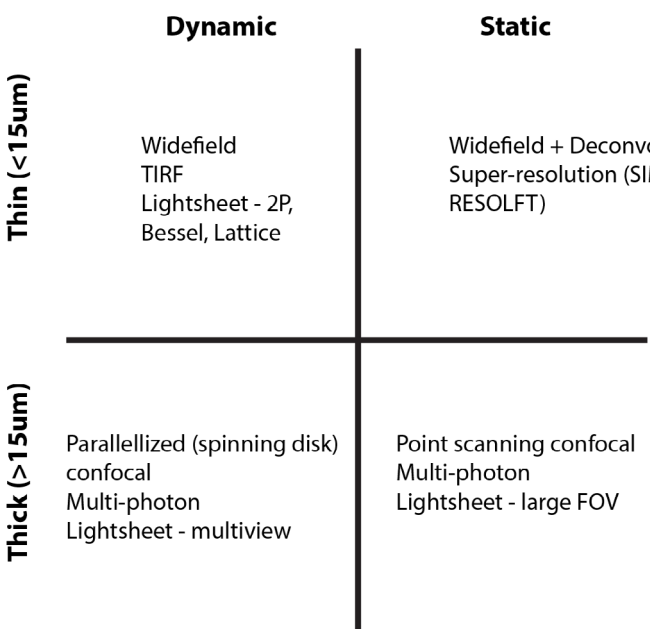
Light Sheet Microscopy – typically utilizes a configuration of two or more objectives to create a thin sheet of excitation light that propagates perpendicular to an imaging objective that collects fluorescence. Like 2-photon microscopy, only a single focal plane of the sample is excited at one time, limiting photobleaching. Similar to widefield, the entire field of view is excited at one time and captured in a single camera exposure. This is much faster than relying on raster scanning as is done in confocal or 2-photon microscopes.

Total Internal Reflection Microscopy (TIRF) – is a technique used to only excite a very thin layer of fluorescent molecules sitting right next to the coverslip. Light is projected through the coverslip at an angle such that when it reaches the interface between the glass coverslip and a sample in aqueous buffer it is completely reflected. This reflection occurs due to the refractive index mismatch between the glass and the water-like buffer the sample is immersed in. Although the excitation light is completely reflected, energy is propagated into the sample via an evanescent wave that only excites fluorophores within a few hundred nanometers of the glass/water interface.

Super Resolution Microscopy – allows for imaging below the diffraction (resolution) limit of a light microscope. Due to the wave nature of light, an infinitely small point of light will blur to a 200-300 nm sized spot while passing through the optics of a microscope before it reaches the detector. This means that two or more objects lying within the diffraction limit will appear as one object in the final image. Over the past two decades a number of techniques have been developed that allow for sub-diffraction limit imaging. Most often, these techniques provide a 2-10x improvement in the resolution of a light microscope (see image above, right).

FLUORESCENCE MICROSCOPY TECHNIQUES (CONT'D)

What Microscopy Technique Should I Use for My Experiment?



When I am contemplating which microscope to use for a new sample I always start by asking two questions:

1) Is the sample dynamic or static?

2) Is the sample thin (< 15 µm) or thick?

These questions will place each sample into one of four categories that are each suited to different types of modern day fluorescence microscopes (see Table 1).

Thin Dynamic Samples

Ex: monolayers of live cells with fluorescently labelled, motile structures

These samples are thin enough to sit within the depth of field of the objective (the distance in the axial dimension that will appear in focus). This means that the images will appear sharp without interference from blurry out-of-focus light. The most difficult characteristic to capture in these types of samples is their fast movements. Depending on the sample, a temporal resolution on the millisecond scale may be needed. Most commonly these experiments are performed on a fluorescent widefield microscope. Here, excitation light is projected from the objective so that the entire field of view is bathed with an even illumination. The emitted fluorescence from all dye molecules in the sample is then collected and projected back to a fast, sensitive detector such as a scientific CMOS camera.

If plasma membrane dynamics are the main focus of the experiment, a Total Internal Reflection Fluorescence (TIRF) microscope can be used. TIRF only excites fluorophores within a few hundred nanometers of the coverslip (4). Essentially, an optical section is achieved that boosts contrast by filtering out emission light from fluorophores higher in the sample. TIRF is also very gentle on the sample as the majority of the laser light is reflected away from the sample and excitation occurs through the fluorophore's interaction with an evanescent energy field.

Lightsheet microscopes that project ultra-thin sheets of excitation light through multiple focal planes of a sample may also be useful for providing fast imaging along with optical sectioning (5). One such example is the lattice lightsheet that projects a number of thin cone-shaped beams into the sample. These beams interfere to form a single plane of excitation light that is thinner than the portion of the sample that is in focus. This technique can be used to provide high 3D spatial resolution while also avoiding the toxic effects of high light doses to the cells.

Thin Static Samples

Ex: Fixed monolayers of cells or thin (<15 µm) tissue sections and 3D cultures

FLUORESCENCE MICROSCOPY TECHNIQUES (CONT'D)

These samples do not necessarily require optical sectioning, but open the door to a number of other techniques that can improve contrast and resolution due to the lack of a requirement for high temporal imaging speeds. Standard fluorescence widefield microscopy can be combined with deconvolution, a mathematical software post-processing step that reassigns out of focus light to its respective focal plane. In sufficiently thin samples, deconvolution can outperform optical sectioning techniques due to a higher photon flux via the more efficient camera detector and lack of a light-reducing pinhole. By collecting more photons, a higher signal to noise ratio can be achieved (6).

Super-resolution techniques such as Structured Illumination (SIM), Single Molecule Localization (PALM/STORM), or Reversible Saturable Optical Linear Fluorescence Transitions (RESOLFT/STED) also excel on these types of samples. Prior to the advent of super-resolution imaging, the light microscope was limited in its capacity to resolve closely lying structures. This is because light that passes through the optics of a light microscope is diffracted. Even an infinitely small point of light – think of a single GFP molecule – will appear as a fuzzy 200-300 nm spot when imaged. Super-resolution microscopy uses a number of optical and chemical 'tricks' to turn subsets of fluorescent molecules on and off. This can provide a 2-10 fold increase in resolution, down to 10s of nanometers.

Thick Dynamic Samples

Ex: 3D cell cultures, small model embryos >15 µm

Thsee are some of the most challenging samples to image as they require both optical sectioning, imaging over large distances and high temporal resolution. Traditionally, this type of sample is imaged with a parallelized confocal technique such as spinning disk confocal. However, due to the scattering nature of biological tissue, imaging more than 80-100 um in depth may not be possible. Therefore, 2-photon microscopy which relies on deeply penetrating infra-red excitation light can push imaging depths closer to 1 mm. Traditionally, 2-photon has been a very slow imaging technique but recent advances in scanner technology and parallelization (7) have allowed for real-time monitoring of neuronal activity across substantial 3D volumes.

In addition to 2-photon microscopy, lightsheet microscopy is rapidly becoming the preferred technique for many of these samples due to its fast temporal resolution and decreased phototoxicity. Lightsheet designs that allow for multi-view imaging can image thick scattering samples in their entirety, something that is not possible via traditional confocal due to limitations in light penetration mentioned above (5).

Thick Static Samples

Ex: Fixed tissue sections (> 15 um), 3D cultures, and cleared tissues

These samples always require some form of optical sectioning. Point scanning confocal microscopes (see above) often provide the highest quality images for this type of sample as they are most efficient at excluding out of focus light. However, they also continually dose the sample from top to bottom with excitation light which can lead to photobleaching when acquiring multiple images over a large axial range. 2-photon imaging, which restricts excitation and photobleaching to the focal plane can be used to overcome this (8).

The recent development of tissue clearing techniques (9) now allows researchers to image tissues that are over a cm^3 in size. Point scanning confocal and 2-photon microscopes which rely on building an entire image

FLUORESCENCE MICROSCOPY TECHNIQUES (CONT'D)

pixel by pixel are unable to provide the frame-rates necessary for imaging tissues of this size. For example, it could take nearly two months to image an entire mouse brain with a 20x/1.0NA objective with proper sampling. Therefore, large, cleared tissues need to be imaged via Lightsheet microscopy. Although not all lightsheet microscopes can cover samples of this size, those that can will completely image large tissues in a few hours.

Although the modern explosion in light microscopy techniques has provided biologists with a host of tools and opened doors to many great insights, it can be overwhelming to a novice microscopist. Hopefully this brief synopsis can help point you in the right direction. You certainly shouldn't be afraid to seek out expertise from a well-established microscopist at your institution when heading into a new set of experiments.

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LIGHT SHEET FLUORESCENCE MICROSCOPY

By Jae Lee and Pentelis Tsoulfas | August 26, 2015

The beginning of this century has seen some major advances in light microscopy, particularly related to the neurosciences. These developments in microscopy coupled with techniques that make tissues transparent are enabling microscopes to visualize the cellular architecture of whole tissues in 3D with unprecedented detail. One of these advances in microscopy has been light sheet fluorescence microscopy (LSFM). The underlying method was developed in 1902 by Richard Zsigmondy and Henry Siedentopf to enhance the microscopic resolution for studying colloidal gold (1). The method was based on using a thin plane (sheet) of light generated by sunlight to observe single gold particles with diameters less than 4 nm.

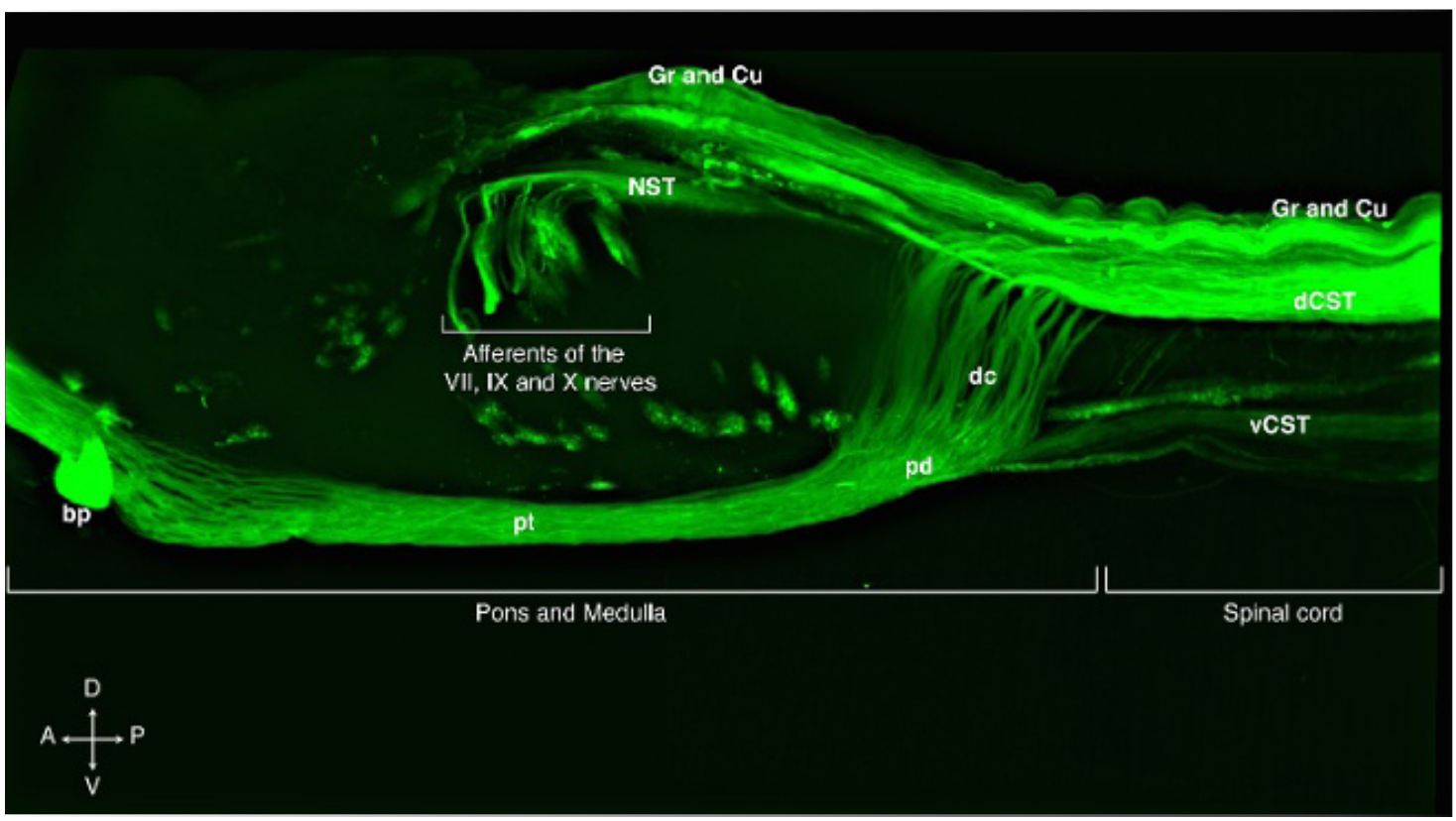

Figure 1. Lateral view of a cleared rat pons, medulla and the first segments of the spinal cord showing the cortico spinal tract, CST, spinal cord and cranial nerve afferents labelled with a AAV8 expressing eGFP under the human ubiquitin C promoter . Anatomical abbreviations: Gr= Gracile tract, Cu= Cuneate tract, dCST= dorsal cortico spinal tract, vCST= ventral cortico spinal tract, dc= decussation of the CST, pd= pyramid, pt= pyramidal tract or CST traversing pons and medulla, bp= basilar pons, NST nucleus of the solitary tract. The arrows point the direction of the specimen, A= anterior, P= posterior, D= dorsal, V= ventral

Developments in Light Sheet Fluorescence Microscopy

Modern light sheet fluorescence microscopy was first pioneered by Voie and colleagues and originally named orthogonal-plane fluorescence optical sectioning (OPFOS) (2). Arne Voie, David Burns and Francis Spelman focused a laser beam into a thin sheet to illuminate a fluorescent sample and captured the reflected light using a different objective lens oriented perpendicular to the plane of illumination (i.e. light sheet). This is in contrast to confocal laser microscopy where the laser and the reflected light travel through the same objective lens. Using this method, these authors were able to reconstruct a cleared guinea pig cochlea in 3D. The basic configuration of the instrument used in this reconstruction lay the foundation for all subsequent versions of LSFM microscopes (3,4).

LIGHT SHEET FLUORESCENCE MICROSCOPY (CONT'D)

After this initial application, Stelzer's group described the single-plane or selective-plane illumination microscope (SPIM). In order to improve axial resolution and reduce phototoxicity for imaging live specimens, they combined light sheet microscopy with sequential multiple-view reconstruction (5). In order to image large tissues such as cleared rodent brain, the Ultramicroscope was developed using a stereomicroscope to capture large field-of-views (6). For more comprehensive reviews on other LSFM configurations' features see (4,7).

Light Sheet Fluorescence Microscopy vs. Confocal Microscopy

In confocal microscopes, optical sectioning of a specimen is based on discriminating the out-of-focus reflected light by using a pinhole. However, the excitation light excites all of the fluorophores as it passes through the specimen, which often leads to photobleaching. Furthermore, point-by-point scanning along the entire specimen makes imaging too slow and unwieldy for large specimens that are millimeters in thickness (8,9). In addition, there is a drop in image brightness with increasing depth caused by light scattering and absorption. Finally, the photomultipliers used to detect light in confocal microscopes are less efficient than the modern cameras used in LSFM.

In LSFM the laser light sheet, typically 2-6 microns, illuminates only one thin plane of the sample surrounding the focal plane of the detection lens and thus there is no out of focus light. Therefore, there is much less photo-bleaching or photo-damage than in conventional laser scanning microscopy. Since image acquisition is performed using cameras with electron multiplying couple-charged device (EMCCD) or complementary metal-oxide semiconductor (CMOS) sensors, it takes only a few milliseconds to acquire one image, thereby significantly reducing the time it takes to image through a large z-stack (8). One limitation of LSFMs using single side illumination, especially with large tissues, is that any obstacles (e.g. air bubbles or a high concentration of fluorophores) cast shadows along the illumination path. The shadows appear as stripes in the acquired image (5). A way to get around this problem is to illuminate and acquire images of a sample from two opposing sides and merge the images, such as in the Ultramicroscope. In addition, adjusting the thickness of the laser sheet and using multi-view imaging can provide enhanced resolution (8,9). Thus, LSFM is ideal for 3D reconstruction of clear specimens and for *in vivo* imaging of transparent organisms.

Advances in Tissue Clearing

Similar to light microscopy, there have been significant advances in tissue clearing techniques during this century. The first successful attempt to render fixed anatomical preparations transparent was achieved by Walter Spalteholtz (10). He used a solution of benzyl alcohol and methyl salicylate which was later modified by others to produce Murray's clear solution. Murray's clear (11) was mostly used to study the development of vertebrate embryos. The first 3D reconstructions of clear embryos were obtained by using optical projection tomography (OPT) (12) and later Hans-Ulrich Dodt's lab pioneered the use of light sheet microscopy on cleared whole-mount specimens, such as whole mouse brains expressing green fluorescent protein (GFP) (6).

The Dodt and Frank Bradke laboratories later developed the 3DISCO (3-dimensional imaging of solvent cleared organs) method of imaging rodent brains and spinal cords by combining Ultramicroscopy with tetrahydrofuran-based tissue clearing (13,14). A few years later, Karl Deisseroth's lab developed CLARITY, which is a hydrogel-based method that also allows antibody penetration for immunohistochemical labeling in whole tissue (15). CLARITY is also compatible with LSFM, a method termed COLM (clarity-optimized light-sheet microscopy) (16). Both 3DISCO and CLARITY have relied on the use of transgenic mice in which a subpopulation of neurons are brightly labeled with GFP (e.g. Thy1-YFP-H mice). However, we recently developed AAV (adeno-associated virus)-based fluorescent labeling methods that can be used with 3DISCO to image non-transgenic animals such

LIGHT SHEET FLUORESCENCE MICROSCOPY (CONT'D)

as rats (17). In addition, the use of viruses allows much better spatiotemporal control over labeling methods as compared to transgenic animals (17). These viral plasmids are available through Addgene. The combination of LSFM with multiple tissue clearing strategies and neuronal labeling methods will greatly aid our understanding of the structure-function relationship of the central nervous system under normal and diseased conditions.

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CHAPTER 2: GENERATING FLUORESCENT PROTEIN FUSIONS

THE MICHAEL DAVIDSON PLASMID COLLECTION

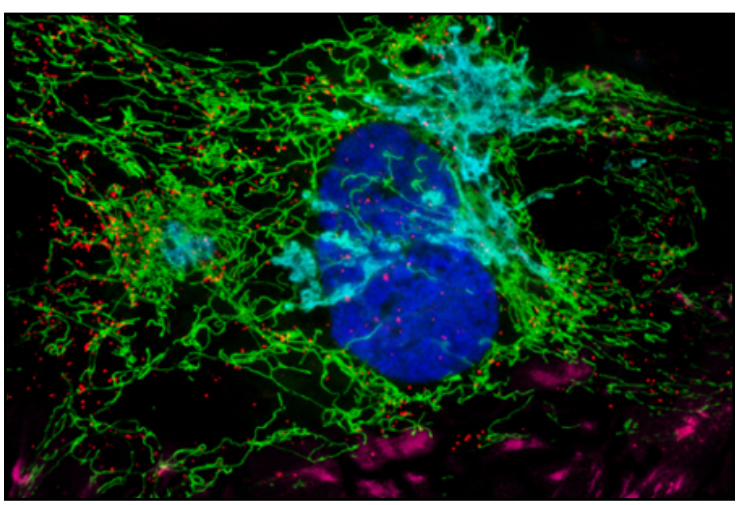
By Lianna Swanson | Mar 17, 2017

This chapter will discuss many different ways you can fuse your gene of interest to a fluorescent protein. Among many other possibilities, these fusions can help you monitor the expression and localization of your protein of interest (although you should check out our chapter on Fluorescent Protein Pitfalls and make sure your fusion functions properly first!). We'll cover techniques for tagging genes both *in vitro* and *in vivo* and start by discussing the Davidson Plasmid Collection which contains many vector backbones you can use to tag your gene of interest in addition to many vectors with pre-tagged cellular markers.

If your favorite method for generating fluorescent protein fusions is missing, be sure to reach out to us at blog@addgene.org and we'll try to include the method in a future blog post and in the next update to this eBook.

Michael Davidson (1950-2015) dedicated his scientific career to 3 major avenues – mentoring young students and instilling a strong work ethic in them, developing educational resources for microscopy, and building new fluorescent protein tools for the scientific community. Davidson took the fluorescent proteins originally developed by Roger Tsien, a frequent collaborator, and expanded on them to revolutionize the study of cell biology. In 2014, Mike Davidson deposited his plasmid tools with Addgene.

The collection consists of over 3000 plasmids, with the vast majority in mammalian expression backbones and a few constructs in a bacterial expression backbones. These plasmids can be divided into the following categories:

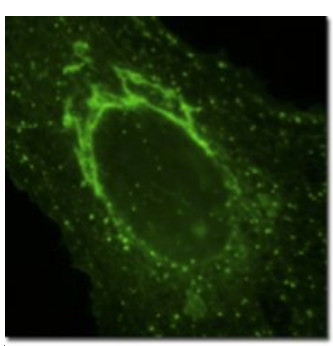


Cells labelled with mTurquoise-Golgi, mVenus-Nucleus, and mCherry-Mitochondria. Image credit: Michael W. Davidson and the Florida State University Research Foundation.

- A comprehensive list of cell markers all tagged with mEmerald.
- Over 300 empty backbones with 100+ distinct fluorophores to chose from in the green, red, yellow, cyan, blue, and orange spectra.
- Plasmids to monitor protein-protein interactions through multicolor labeling and FRET.
- Specialty constructs for biosensing.

This post will discuss the first three categories in detail, but you can find out more about biosensors here.

mEmerald Tagged Cell Markers

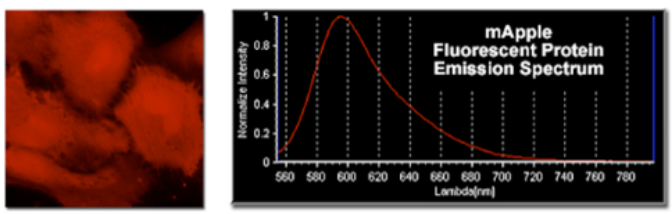


Fluorescent tags can be used to monitor protein localization, interactions, and translocation. They can also be used to measure relative expression levels by comparing fluorescence intensity to that of other known factors. If you are a novice in microscopy techniques or are in need of a visual representation of various cell components for educational purposes, the comprehensive mEmerald-tagged collection is your best friend. From F-Actin to mitochondria, from the nucleus to cell membrane, this collection has fluorescently tagged versions of many proteins known to localize to specific cellular structures. The Davidson lab even included some of their own reference images that can be found on the corresponding

THE MICHAEL DAVIDSON PLASMID COLLECTION (CONT'D)

plasmid pages (e.g. see Supplemental Documents here). If you're not sure where your protein of interest localizes in the cell, use these markers as your guide.

Empty Backbones



mApple Expression in HeLa Cells (left) and emission spectrum (right).

While the mEmerald collection can show you specific cellular structures, to determine whether or not your protein of interest colocalizes with one of these markers, you'll need to fuse it to its own fluorescent protein. The empty backbone collection enables you to do just that. You can chose one of the many backbones to clone your gene of interest into and visualize it in cells. The Davidson collection has the classics (e.g., EGFP), the new and improved fruit colors (e.g., apple, papaya, and tomato) as well as photoactivatable fluors; many of these fluorescent proteins have been specifically mutated to improve their brightness and stability. When choosing a backbone to clone into, please consider the following points:

1. **Ensure your cDNA of interest and the fluorescent tag are co-expressed properly:** double check the DNA sequence to make sure that they are in the same reading frame and there are no accidental stop codons between them.
2. Since the fluorescent proteins are relatively large, it is recommended that you **include a ~7-9 amino acid glycine and proline linker** between the fluorescent protein and your protein of interest. This should allow for proper folding of the fluorophore and your protein of interest.
3. **Test N and C-terminal tags.** Tagging the N or the C terminus of your protein of interest with a fluorescent protein may affect its function or localization, so you may need to experiment with both. Luckily most of the Davidson backbones come with both options, having convenient MCSs (multiple cloning sites) on either side of the fluorophores. (Ex. mApple-C1 and mApple-N1).
4. **Consider whether you are planning on expressing multiple fluorescently tagged proteins and visualizing them in the same cell.** To do so you will need to make sure that the assortment of fluorescent proteins (from different backbones) have sufficiently different excitation and emission spectra for them to be imaged in distinct fluorescent channels on the microscope. Read more about multicolor imaging.

Protein-protein Interactions

The Davidson collection is an important tool for studying protein protein interactions within the cell. Many of the fluorescent proteins within the collection are spectrally distinct enough to be used to observe separate proteins in the same experiment. Observing colocalization of two differentially labeled proteins, a scientist can analyze correlation coefficients to determine whether the spatial overlap is a result of co-occurrence or correlation; where the former is due to two (possibly unrelated) fluorophores occupying the same pixel, and the latter indicates a more significant statistical relationship between the fluorophores suggesting biological interaction (Dunn et al 2011). This can be accomplished in live cells or via traditional fixing and immunohistochemistry methods.

Another popular and widely used approach to study protein-protein interaction using fluorescent proteins is FRET, Förster or fluorescence resonance energy transfer. In FRET, the energy relaxation of an excited donor fluorescent protein is emitted through non-radiative transfer to a nearby acceptor molecule instead of being emitted as light. The energy transfer is limited to distances less than 10 nanometers and the efficiency of

THE MICHAEL DAVIDSON PLASMID COLLECTION (CONT'D)

transfer is inversely proportional to the sixth power of the distance between donor and acceptor - only really closely interacting proteins should generate a FRET signal. Due to the sensitivity and distance requirements for FRET to occur, this technique can be helpful in determining whether two proteins interact directly or are simply in the same vicinity. The Davidson lab has deposited several ready to use FRET constructs to speed up your protein protein interaction studies.

I invite you to browse and utilize this great collection for your current and future cell biology studies, it has everything you need to get going - markers for specific cellular structures, backbones for easy fusion protein generation, and a spectrum of colors for multicolor imaging. If you use plasmids from this collection to create your own fluorescent protein fusions, we encourage you to deposit them back with Addgene and thereby expand the repertoire of fluorescent protein tools available to your colleagues.

Further Reading

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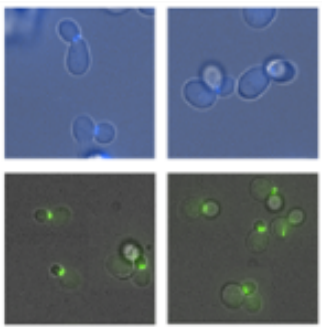
TAG YOUR FAVORITE YEAST GENES WITH EASE

By Julian Taylor-Parker | November 19, 2013



Modified from Lee S, et al. (2013) PLoS ONE 8(7): e67902.

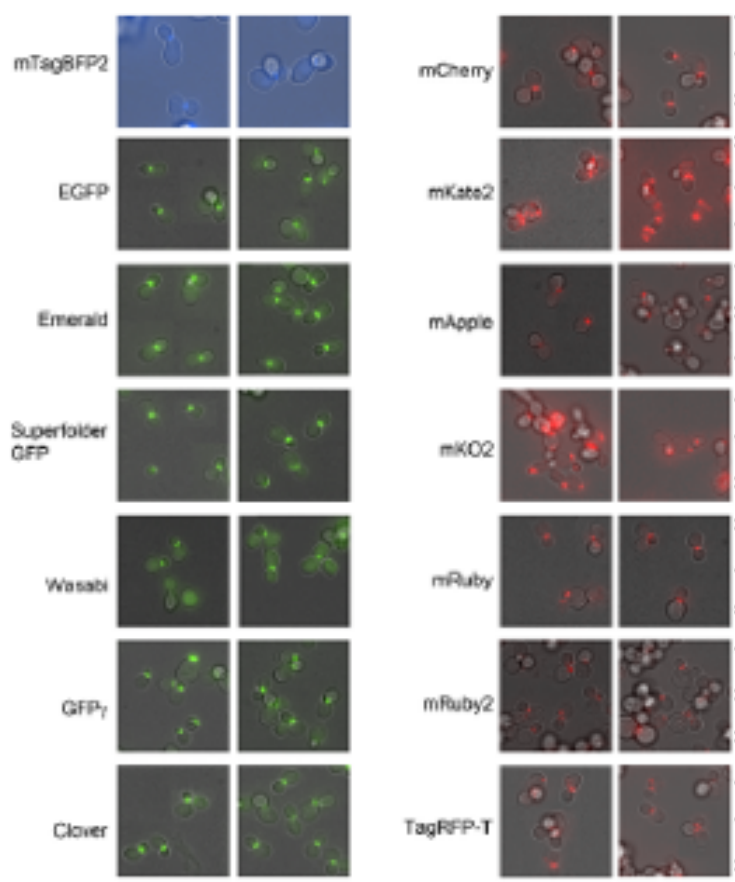
Homologous recombination is the process by which nearly all domains of life repair genomic damage, specifically double strand breaks. Researchers have long taken advantage of this natural process to **integrate protein tags into the genomes of *S. cerevisiae* and *S. pombe***. The protocol is surprisingly simple, requiring only a PCR product containing the modifying sequence flanked by approximately 50 base pairs of sequence homologous to the chromosomal site of insertion. The linear PCR product is introduced into the cell by direct transformation. A given insert will typically contain both a protein modification sequence and a selectable gene product for isolation of successful transformants.

Addgene distributes several ready-to-use, modular plasmids, combining fluorescent tags, epitope tags, protease sites, and selection markers. These are especially useful in protein complex studies where tagging of multiple protein products is desired, as multiple selection markers can ensure that all desired tags have been integrated. Simply design your amplification primers with the desired targeting homology—in frame, of course—and start tagging!

Yeast-Optimized Fluorophores for Imaging

Many imaging studies rely on **direct fusion of fluorescent proteins (FPs)** to a yeast gene of interest. These fluorescently tagged genes are expressed under native conditions and allow scientists to not only track the abundance, movement, and localization of individual proteins, but also investigate protein-protein interactions via FRET. Sidae Lee, Wendell Lim, and Kurt Thorn at UCSF have recently developed a series of blue, green, and red FPs that are codon optimized specifically for expression in yeast. These tagging vectors are based on previously described pFA6a-link vectors and include a Kan, SpHIS5, or CaURA3 selection marker. Lee et al assessed many of these fluorescent tags in *S. cerevisiae*, looking at their performance in categories such as brightness, stability, and disruption of the tagged protein. Based on their findings, the authors recommend optimal FP combinations for use in yeast imaging, categorized by specific filter sets and experimental output requirements. Select from these yeast-optimized fluorophore tagging vectors for your single or multi-color imaging experiments.

If you're looking for a great resource about imaging techniques, check out Kurt Thorn's microscopy blog.



Lee S, et al. (2013) PLoS ONE 8(7): e67902.

TAG YOUR FAVORITE YEAST GENES WITH EASE (CONT'D)

Interested in Epitope Tags?

Others may be interested in **attaching epitope tags** to their genes of interest, allowing for easy capture and detection of proteins and complexes, without the artifacts sometimes associated with plasmid-based overexpression. Tim Formosa, at the University of Utah, has built a complete collection of yeast tagging modules with each possible combination of protease site (TEV or PreScission), epitope tag (12xHis, 2xStrep, 3xFlag, Protein A, or V5), and selection marker (KanMX, HphMX, or His3MX). Each PCR product from this collection will yield an insert with the format (protease site)-6xGly linker-(epitope tag)-ADH1 terminator-(selection marker). Additionally, Dr. Formosa has deposited six plasmids with a multiple cloning site in place of the epitope tag for creation of your own unique protein fusions. This collection is ideal for **tandem affinity purification** of protein complexes.

John Pringle and Jürg Bähler have deposited a large collection of plasmids for genome modification in yeast developed by Dr. Pringle's former lab at UNC Chapel Hill. Bähler et al describe a modular collection of plasmids for a wide variety of genome modifications in *S. Pombe*, including full and partial gene **deletion, overexpression** (by promoter substitution), and **tagging** at either the N- or C-terminus (3xHA, 13xMyc, GST, or GFP). Longtine et al describe a complimentary set of plasmids for use in *S. cerevisiae*, with the additional benefit of multiple selection markers for combining modifications within a single strain.

In addition to the collections featured above, many other modular yeast tagging systems have been developed in the labs of Anne Robinson, Eishi Noguchi, and Melissa Moore, to name a few.

Have you used these tools in your own lab? Addgene would love to hear from you, our community, about your experience with yeast genome modifications. In what ways have genome tagging systems enabled you to advance your research? Do you have a favorite tagging system that isn't mentioned here?

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MXS-CHAINING

By Leila Haery | February 7, 2017

High-throughput cloning, in a nutshell, is the systematic combination of different genetic sequences into plasmid DNA. In high throughput cloning techniques, although the specific sequences of the genetic elements may differ (e.g., a set of various mammalian promoters), the same cloning procedure can be used to incorporate each element into the final construct. This strategy can be used to build vectors with diverse functions, and thus, is used in many biological fields. In synthetic biology for example, high-throughput cloning can be used to combine the functions of different genetic elements to generate non-natural tools such as novel biological circuits or sensors. Given the expanding palette of fluorescent proteins and the availability of powerful imaging technologies, the combination of multiple fluorescent protein sequences to develop diverse fluorescent reporters is a useful application of high-throughput cloning. MXS Chaining is one such technique and has been used to produce complex fluorescent reporter constructs. These fluorescent reporters can be used to detect structure and protein localization, as well as cellular processes like gene expression and cell migration (Sladitschek and Neveu, 2015).

Origin and Purpose of MXS Chaining

MXS-chaining was designed to create plasmids for fluorescence imaging or flow cytometry applications in mammalian cells. The modules used in this method include fluorescent proteins, promoters, enhancers, polyadenylation signals, inducible gene-expression sequences, and more. By combining these components, one can generate constructs to visualize cell cycle dynamics, titrate inducible transgene expression, or a variety of other clever applications.

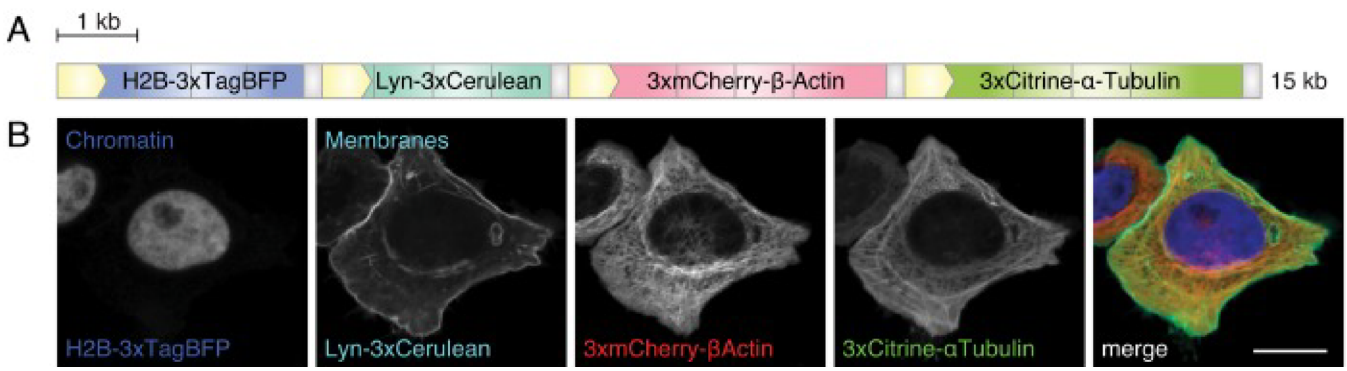


Figure 1: Fluorescent protein expression from an MXS chaining construct in HeLa cells. Figure adapted from Sladitschek et. al., 2015.

In one example, modular constructs generated with MXS-chaining were used to label subcellular structures in live cells. Four individual expression constructs were created, each containing a unique fluorescent protein with minimal spectral overlap between them (Table 1). In each construct, 3 copies of the fluorescent protein were fused in-frame to a tethering partner, which directed localization of the fluorophore, and thus enabled labeling of particular structures (Table 1). Each construct was flanked with a CMV promoter (to drive high-level expression) and a polyA signal (to terminate transcription) and the four constructs were then combined to create a single polycistronic 15 kb insert. The final construct was then introduced to HeLa cells (Figure 1). The resulting HeLa cells show robust labeling, with strong expression and detection of each fluorophore at the respective subcellular structure (Figure 1). Here, MXS Chaining gave researchers the ability to generate the four initial unique constructs and later combine all four constructs into one 15 kb insert.

MXS-CHAINING (CONT'D)

Table 1: MXS Constructs for Labeling Cellular Structures

Construct	Fluorophore	Excitation/ Emission Maxima	Tethering Partner	Subcellular Localization
1	TagBFP	399nm/ 456nm	histone 2B (H2B)	Chromatin
2	Cerulean	433nm/ 475nm	Lyn-tag (derived from the tyrosine-protein kinase Lyn)	Membranes
3	mCherry	587nm/ 610 nm	human β-Actin	Actin
4	Citrine	516nm/ 529nm	human α-Tubulin	Tubulin

Repeatable, Directional Cloning

The principle behind MXS-chaining is repeatable, restriction-enzyme based (chaining-based) cloning. The modules (Figure 2) are contained in individual plasmids and are each flanked by the same multiple cloning site (MCS). Modules are assembled one at a time by standard restriction enzyme digestion followed by ligation. Upon ligation, the original pattern of the MCS is regenerated at the flanks of each ligation product, which is the basis for the repeatability of the method. Note that the restriction sites of the MCS are only regenerated at the 5' and 3' termini of the ligation product, and the restriction site between the modules is eliminated upon ligation. As a result, once two modules are assembled next to each other, a third module cannot be placed between them, but rather, can only be added to the 5' or 3' terminus using the newly generated MCS. MXS-chaining also has the advantage of being directional, meaning that (in the previous example) one can control whether the third module is added to the 5' terminus or the 3' terminus of the ligation product.

Comparison to Other High Throughput Cloning Strategies

There are several high-throughput cloning strategies available for assembling these modular types of constructs, and different methods are suited for particular downstream applications. The tradeoffs between various cloning methods are listed in Table 2. For example, chaining-based cloning methods require that the separate modules combined using

Contents of the MXS-Chaining Kit

6 chaining vectors with multiple cloning sites

14 fluorescent proteins

TagBFP, Citrine, E2-Crimson, Cerulean, mKO2, iRFP670, LSS-mKate2, tdTomato, PA-GFP, mEGFP, mCherry, Kaede, mAzamiGreen, mPlum

10 promoters & enhancers

CMV, PGK, EF1a, Tet, minCMV, minPGK, CAG, BiTet, CMV enhancer, PGK enhancer

3 polyA signals

bGpA, bGHpA, SV40pA

miscellaneous

loxP, H2B, P2A, PEST2D, Gly-Ser—rich linker

tools for inducible gene expression

CMV::CreERT2-bGHpA, PGK::CreERT2-bGHpA, CMV::rtTA3-bGHpA, PGK::rtTA3-bGHpA, CMV::tTA2-bGHpA, PGK::tTA2-bGHpA

8 selection cassettes

CMV::HygroR-bGHpA, PGK::HygroR-bGHpA, CMV::NeoR-bGHpA, PGK::NeoR-bGHpA, CMV::PuroR-bGHpA, PGK::PuroR-bGHpA, CMV::ZeoR-bGHpA, PGK::ZeoR-bGHpA

Figure 2: Contents of the MXS chaining kit.

MXS-CHAINING (CONT'D)

these techniques are free of specific restriction enzyme recognition sites. Thus, chaining methods are not necessarily suitable for combining endogenous sequences.

Table 2: High Throughput Cloning Strategies

Method	Technology	Pros	Cons	Refs
Golden Gate	Type II restriction enzymes	Can assemble up to 10 modules in a single step. Through iteration of this step, the final construct can contain an unlimited number of modules	Constraints on the order of the modules, because neighboring fragments require compatible cohesive ends	Engler et al., 2008
Gibson Cloning	Gibson assembly method	Single reaction, can assemble large DNA sequences, no restriction enzymes needed	Not optimal for joining sequences with a high degree of identity, so not suited to generate polycistronic constructs containing multiple identical polyadenylation signals or promoters	Gibson et al., 2009
MXS-Chaining, BioBricks, Bglbricks, etc	Chaining-based methods (restriction enzyme-based)	Well suited for the modular assembly of highly similar or repetitive sequences. Intermediate chaining products (called "cassettes") can be readily reused in the assembly of any later construct (i.e.,functional cassettes are recyclable)	Can only work if the restriction sites used for the assembly are not found within the modules themselves (may not be suitable for endogenous coding sequences), so enzyme choice is critical. May not support in-frame fusions of coding sequences	Sladitschek and Neveu, 2015; Shetty et al., 2008; Anderson et al., 2010

Among the chaining-based methods, the specific restriction enzymes used affect what general types of modules can be assembled. For example, because CpG dinucleotides are underrepresented in most vertebrate genomes (Josse et al., 1961, Swartz et al., 1962), SalI, XhoI and MluI recognition sites, which contain CpG dinucleotides in their 6-mer sequences, are rarely found in the transcriptomes of these species and can often be used to clone cDNAs from them. These enzymes might not, however, be appropriate for cloning genes from organisms with high CpG dinucleotide representation. The specific restriction enzymes used in various chaining-based cloning methods are listed in Table 3.

The goal of high throughput cloning is to facilitate the construction of plasmids with many components. The creators of the MXS-chaining method have used the technique to engineer various constructs with applications in flow cytometry approaches in mammalian cell culture systems (their original goal). Their work has demonstrated that the MXS-chaining method can be used to build these types of constructs robustly, quickly,

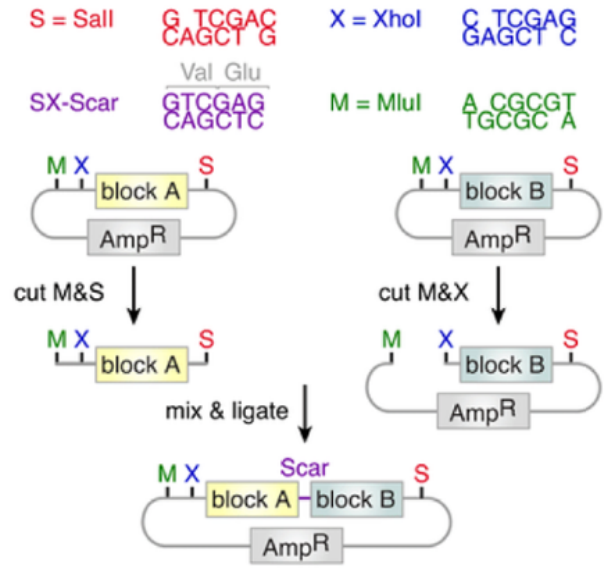
MXS-CHAINING (CONT'D)

and simply. We hope that you can similarly apply MXS chaining to your experimental needs. Let us know how you use MXS Chaining by emailing us at blog@addgene.org.

Table 3: High Throughput Cloning Strategies

Method	Restriction Enzymes	
	Chaining	Direction
MXS - Chaining	SalI and XhoI	MluI
BioBricks	SpeI and XbaI	EcoRI and PstI
BglBricks	BglII and BamHI	EcoRI

Principle of MXS-Chaining



Example Application

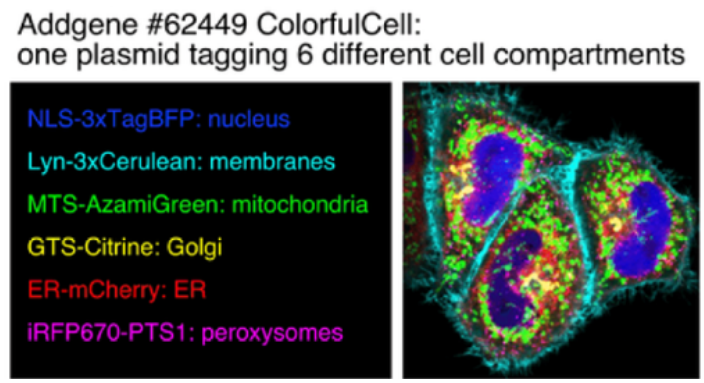


Figure 3: Principle of the MXS chaining method.

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SUNTAG

By Mary Gearing | Mar 28, 2017

In biology as in life, more is often better. More transcription factor binding sites in a promoter lead to higher transcriptional activation. Multiple nuclear localization signals (NLS) increase protein import into the nucleus. In developing their SunTag technology, the Vale and Weissman labs took this biological lesson and created a system to amplify fluorescent signals. Named for the "stellar explosion SUperNova," SunTag can help you turn up the brightness in your fluorescent imaging experiments.

Fluorescent Protein Fusions

One of the easiest ways to track a given protein is to fuse it to a fluorescent protein. You can then study where the protein is localized, and how its localization and expression may change across various conditions. However, this system is far from perfect. For example, If your fusion protein is expressed at low levels, you have to increase your imaging time to get enough signal. This workaround risks cellular phototoxicity and eliminates the possibility of long-term imaging studies. If you instead overexpress your protein at higher levels, you risk observing artifacts only present when the protein is at a very high concentration. Overexpressed proteins also have the potential to form aggregates and may be toxic to the cell.

Here Comes the SunTag

How does SunTag fix these problems? Instead of directly fusing a fluorescent protein to your protein of interest, you instead fuse it to the synthetic SunTag scaffold. This scaffold contains 10-24 copies of the short epitope GCN4. GCN4 recruits GFP fused to the cognate scFV antibody, which is expressed from a separate plasmid. This system amplifies the intensity of the fluorescent signal and enables single molecule tracking within living cells without affecting protein function, thereby creating a single-molecule reporter of intracellular processes. Initially, Tanenbaum et al. observed some GFP aggregation, which they reduced by using superfolder GFP (sfGFP) with the small solubility tag GB1. In SunTag nomenclature throughout the rest of this blog post, GFP refers to sfGFP-GB1.

Tanenbaum et al. examined the power of SunTag for single molecule imaging, finding that plasma membrane-targeted CAAX-SunTag was 18-fold brighter than sfGFP! The high SunTag signal allowed them to cut their laser power by over 80% and still obtain a higher signal-to-noise ratio with a lower photobleaching rate. Given the power of SunTag, they attempted single-molecule imaging deep inside the cell, in the nucleus and cytoplasm. Again, they found that SunTag marked single molecules very effectively - they even managed to track run lengths of the motor protein kinesin across microtubules.

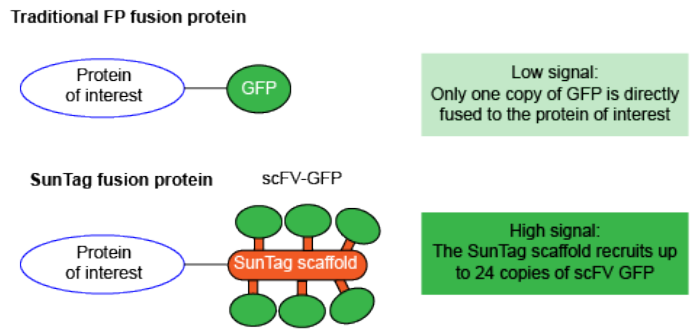

Figure 1: Comparing traditional GFP fusion proteins to SunTag fusion proteins. A traditional GFP fusion uses one copy of GFP attached to a protein of interest. Rather than fuse GFP to a protein, SunTag fusions contain a synthetic scaffold that recruits GFP fused to scFV antibody.

Tanenbaum et al. then tested their hypothesis that the lower expression levels of SunTag constructs would avoid negative effects on cell physiology. Having seen that mitochondrial tracker GFP-mitoNEET can impair mitochondrial function, they examined the effects of mitoNEET-SunTag-GFP. As expected, they obtained bright images of mitochondria without organelle toxicity.

First generation (v1) SunTag is expressed at very low levels due to poor stability of the GCN4 scaffold. To

SUNTAG (CONT'D)

increase expression, Tanenbaum et al. modified the GCN4 sequence to increase its alpha-helical structure and stability, creating v4 SunTag. Since the v4 system does not display protein aggregation, it's recommended for most imaging applications.

What Can You Do with SunTag?

Tanenbaum et al. performed a variety of different experiments in their paper, and so can you! In almost any case you would use a traditional FP fusion, a SunTag fusion could be used as well.

Pros of SunTag compared to traditional FPs:

- Improved brightness and signal-to-noise ratio
- Less chance of phototoxicity
- Reduced photobleaching
- Simplified, long-term single-molecule tracking

Caveats of SunTag

- Very large size: a 24x scaffold fully occupied with GFP has a molecular weight of 1,400 kDa vs 24 kDa for GFP alone. Although traditional FP fusions can also affect protein activity, half-life, or localization, these concerns are greater with SunTag.
- v1 SunTag exhibits some scaffold aggregation (v4 Suntag does not).

It's up to you to determine SunTag's suitability in your experiments on a protein-by-protein basis. However, since many of Addgene's SunTag plasmids have coveted "blue flames," it's clear that the system is broadly applicable across many areas of research. Beyond fluorescence, SunTag can also be used to improve CRISPR-based activation of target genes, but we'll save that application for another day!

Further Reading

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TAGGING GENES WITH CRISPR

By Mary Gearing | December, 2015

First described in the 1980s, protein tags are now one of the most useful items in a scientist's toolbox. As we've covered in Plasmids 101, tags can help you determine localization of a protein of interest, purify it, or determine its expression level without the need for a custom antibody. CRISPR has made it easier than ever to tag endogenous proteins, allowing researchers to track how proteins bind to DNA or to other proteins.

The Basics of CRISPR Taggging

Addgene depositors Eric Mendenhall and Richard Myers have developed a method to easily insert FLAG tags into the C-termini of endogenous loci. To tag your protein of interest, first design a seed sequence targeting the locus you'd like to tag, and then insert it into a plasmid containing Cas9 and the gRNA scaffold. Ideally the gRNA should cut within -5 to +15 base pairs from the stop codon.

Second, create a repair template according to the guide below. Mendenhall and Myers recommend using IDT gBlocks to specify the homology arms of the repair template. Plasmid pFETCh_Donor contains the 3X-FLAG tag and a neomycin resistance gene; the homology arms can be cloned into the vector using Gibson Assembly. Once you introduce the construct into cells, you'll select using neomycin. Isolating clonal populations is not necessary; Mendenhall and Myers use pooled neomycin-positive cells for experiments.

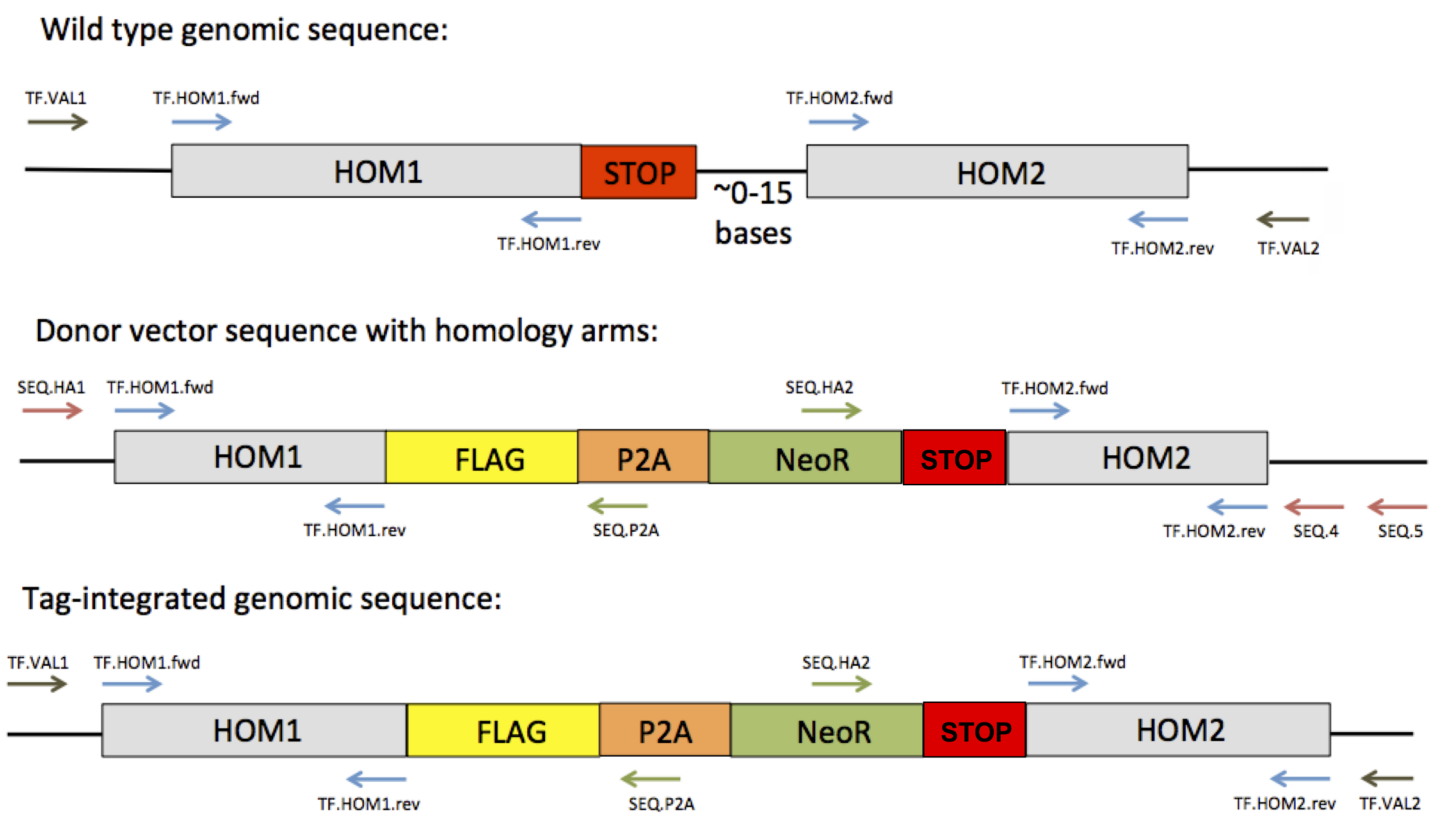

Figure 1: A schematic for FLAG tagging endogenous proteins using plasmid pFETCh_Donor. Left and right homology arms are cloned into the FLAG-P2A-NeoR containing destination vector using Gibson Assembly. Homology-directed repair removes the stop codon and adds a 3X-FLAG tag and neomycin resistance. NeoR is co-transcribed with the FLAG-tagged TF. The P2A self cleaving linker causes the ribosome to skip a peptide bond between the Flag-tagged protein and the antibiotic resistance cassette resulting in two separate protein products.

TAGGING GENES WITH CRISPR (CONT'D)

Application: Multiplexed Transcription Factor Tagging

Mendenhall and Myers are members of the ENCyclopedia of DNA Elements Project (ENCODE), working to define where the ~1,500 transcription factors of the human genome bind. Since fewer than 10% of antibodies are suitable for the ChIP-seq analysis commonly used to map transcription factor binding, they created CETCh-seq (CRISPR epitope tagging ChIP-seq of DNA-binding proteins) to tag transcription factors and analyze their binding in a scalable, global approach.

To test the universality of CETCh-seq, Savic et al. selected five DNA-binding proteins expressed at different levels in HepG2 cultured cells. They designed gRNAs to target the 3' UTR and used an EMM0021-based repair template to add a FLAG tag to the C-terminus of each TF. They screened for homologous recombination using PCR and subsequently verified tag insertion via Western blotting and Sanger sequencing. From the five TFs targeted in parallel, Savic et al. successfully tagged four TFs.

Savic et al. subsequently conducted CETCh-seq using a FLAG antibody. In cells that did not contain a tagged TF, they did not observe binding events, showing that the method has low background. To validate their results, they compared binding from CETCh-seq to datasets obtained using ChIP-seq with verified antibodies, finding an average of ~85% overlap in binding sites between the datasets. Technical and biological CETCh-seq replicates were also highly concordant (rho=0.92-0.98), indicating the robustness and specificity of the technique. Using RNA-seq, Savic et al. verified that TF tagging does not alter the transcriptome, further strengthening the case for CETCh-seq as new method to profile TF binding.

After completing the initial experiments in HepG2 cells, Savic et al. turned to MCF7 cells to verify that CETCh-seq is robust in multiple cell types. For their targeted locus, RAD21, they again found good technical and biological reproducibility, as well as concordance with validated ChIP results. CETCh-seq was also successful in murine embryonic stem cells, opening up the possibility of generating transgenic mice through this tagging approach!

Application: Affinity Purification Tagging for Protein Complex Isolation

Addgene depositor Yannick Doyon is interested in isolating native protein complexes to study biochemical interactions. Dalvai et al. designed a system to add 3X-FLAG-2X-STREP tags to the N- or C-termini of endogenous loci, enabling gentle, high-yield purification of protein complexes. Tagging endogenous loci gets rid of many problems associated with protein overexpression, such as nonphysiological binding. The system can also be used to insert a cDNA into the AAVS1 locus, a "safe harbor" locus that does not interrupt the function of other genes.

To tag genes using the Doyon system, you need a gRNA that cleaves near the terminus of the locus you'd like to tag, as well as a repair template based on Addgene AAVS1_Puro_PGK1_3xFLAG_Twin_Strep. After constructing the homology arms via PCR or with IDT gBlocks, sequentially digest the vector to insert the left arm, then the right arm. A basic schematic is shown below, but further details are available in the supplemental methods of Dalvai et al. If you'd like to use this plasmid to insert a cDNA into AAVS1, just use the multiple cloning site to insert the sequence downstream of the PGK1 promoter.

TAGGING GENES WITH CRISPR (CONT'D)

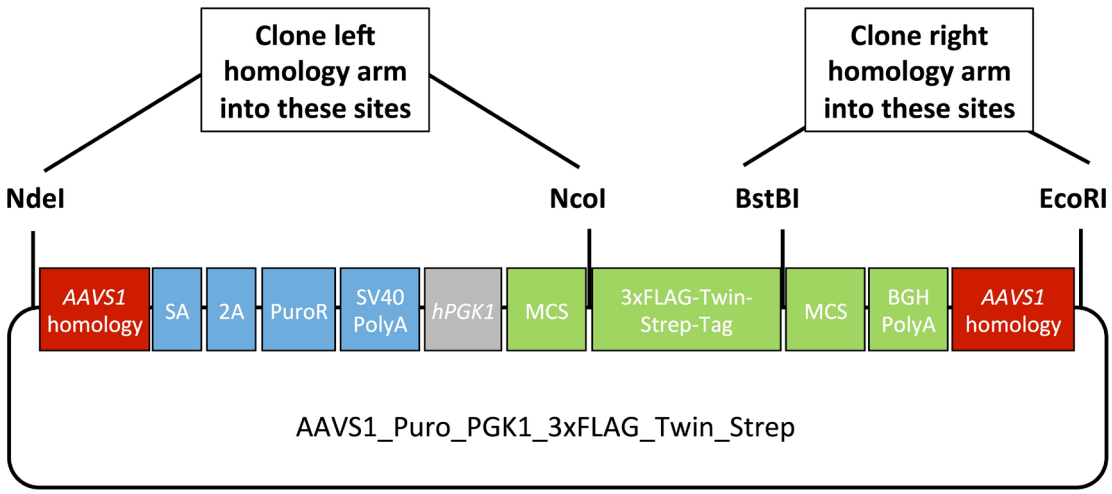

Figure 2: A schematic for 3X-FLAG-2X-STREP protein tagging. To use plasmid AAVS1_Puro_PGK1_3xFLAG_Twin_Strep for endogenous protein tagging, digest with NdeI and NcoI to insert the left homology arm, then digest that construct with BstBI and EcoRI to insert the right arm. If inserting a tagged cDNA into AAVS1, clone the cDNA using the MCS downstream of hPGK1.

Advantages and Tips

These plasmid systems promise to make endogenous protein tagging much easier and faster than ever before. Using gBlocks speeds up cloning, and CRISPR greatly increases the frequency of homologous recombination. Although these systems are FLAG- and STREP tag-based, they can be adapted to other tags, allowing the tagging of multiple loci within a cell population.

One potential pitfall of CRISPR tagging is that the gRNA must bind close to the targeted terminus. If you can't find a gRNA that works for your locus using SpCas9, it may make sense to try alternative Cas9s or Cpf1, which have different PAM requirements.

Ready to start tagging? Plasmids for FLAG and FLAG-STREP tagging are available at Addgene.

Further Reading

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FLUORESCENT TAGGING OF ENDOGENOUS GENES WITH SAPTRAP

By Michelle Cronin | May 16, 2017

Since the discovery of GFP over 50 years ago, the growing spectrum of fluorescent proteins (FPs) has been an invaluable resource for studying the organization and function of cellular systems. FPs have been used to track protein localization, cell structure, intracellular trafficking, and protein turnover rates. Additionally, by engineering FP fusions associated with cellular organelles, scientists have been able to study many cellular processes, including mitosis, mitochondrial fission/fusion, nuclear import, and neuronal trafficking. Although FPs have enabled discovery of many cellular mechanisms, there are some limitations to working with FPs. Overexpression of fluorescently tagged proteins can lead to improper protein localization, protein aggregation, or disruption of normal protein function, and ultimately misinterpretation of the protein's cellular role.

One way to avoid the pitfalls of overexpressed fluorescent protein fusions is to replace the genomic copy of your gene of interest with a fluorescent protein fusion. CRISPR genome editing can help accomplish this goal. The power of this system lies in the ability of the endonuclease Cas9 to create a DNA double stranded break (DSB) at a genomic site specified by a guide RNA (gRNA) sequence. Users can design gRNAs to induce the break at a specific genomic site and, using the endogenous homology directed repair pathway, a new user-defined DNA sequence (like GFP) can be inserted at the DSB. CRISPR has allowed scientists to tag and light up endogenous genes of interest to better understand normal protein function. However, CRISPR protein tagging does have its limitations, especially when trying to perform high-throughput experiments. If a user wants to carry out a high-throughput genetic screen, it is expensive and time consuming to individually generate gRNAs and homology arm repair templates containing the tag for each insertion. Also, it remains challenging to screen for the genetically modified strains that contain your newly tagged gene. Most insertions can only be detected by labor-intensive processes like PCR or by evaluating visual phenotypes.

Improving *C. elegans* Fluorescent Protein Tagging with SapTrap

The Jorgensen Lab recently developed a modular plasmid assembly toolkit called SapTrap to improve CRISPR genomic tagging in *C. elegans*. SapTrap offers scientists a convenient one tube assembly reaction to generate high-throughput targeting vectors. These vectors can be used to tag endogenous genes and simultaneously introduce a selection marker for screening modified strains. With SapTrap, the user first designs either oligos or synthetic DNA for the desired gRNA target sequence, as well as the 5' and 3' homology arm repair template (Fig. 1, Step 1). There is no need for PCR or cloning, as digestion of the destination vector with SapI yields 2 sites- the first site accepts the sgRNA target sequence for U6 promoter expression and the second site accepts the homology arm repair template.

SapTrap includes a prebuilt donor plasmid library containing several types of fluorescent (EGFP, tagRFP, mCherry) and nonfluorescent (Halo, SNAP) tags, a selectable marker (floxed Cbr-unc-119) for easy screening of the insertion event, and a variety of connector modules (linker sequences between the tag and homology arms). Digestion of the donor plasmids with SapI allows the tag, selectable marker, and connector to be released (Fig. 1, Step 2-3). Since donor plasmids of the same type will produce the same unique SapI 5' overhang, different combinations of connector and tag donor plasmids from the library can be used to generate functionally unique repair templates. A final ligation reaction correctly assembles the final targeting vector (Fig. 1, Step 4), and co-injection of the targeting vector and a Cas9 expression plasmid will insert the desired genetic tag and marker sequence into the targeted loci. The selectable marker can be removed by Cre-mediated excision for scarless tag insertion.

In addition to the obvious advantages SapTrap offers for tagging a single genetic locus, the toolkit also offers repair templates for tagging proteins in a tissue specific manner, as well as 3-site destination vectors

FLUORESCENT TAGGING OF ENDOGENOUS GENES WITH SAPTRAP (CONT'D)

for inserting a tag at multiple target sites. SapTrap vectors can be easily modified to tag hundreds of genes, generating powerful genetic screening libraries.

Figure 1: The SapTrap Assembly Method

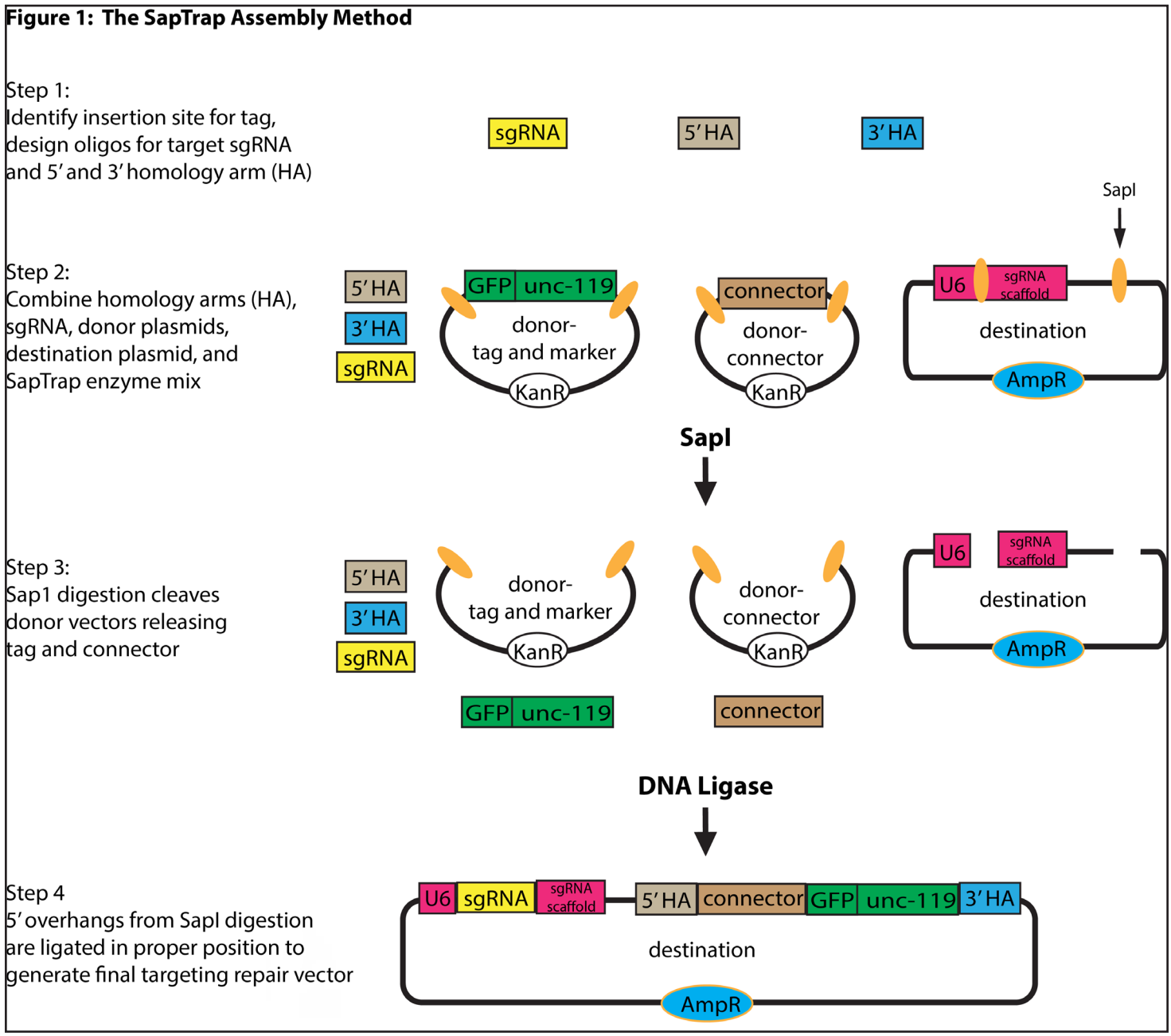

Figure 1: The SapTrap Assembly Method

Additional Protein Tagging Systems

For those who do not work with *C. elegans*, several groups have designed modular toolkits to assist with tagging genomic loci in other model systems, including mammalian cells.

FLUORESCENT TAGGING OF ENDOGENOUS GENES WITH SAPTRAP (CONT'D)

- CRISPaint (CRISPR-assisted insertion tagging), developed by the Hornung Lab, allows users to easily create C-terminal tag fusions of endogenous genes in human cells. To use CRISPaint, users need 3 vectors: 1) a gRNA vector that targets the gene of interest, 2) a plasmid to specify the reading frame of the insertion, and 3) a vector containing the desired tag, which can be obtained as a universal donor plasmid. The CRISPaint toolkit includes several donor plasmids for protein tagging, including luciferase (NanoLuc), fluorescent proteins (TagGFP2, TagBFP, TagRFP, and T2A-TurboGFP-PEST), and small epitope tags (HA, Myc, Strep tag II, AviTag, HaloTag, SpyTag).

- The Foerstemann lab developed a polymerase chain reaction (PCR)-based system that allows users to generate a plasmid containing a direct fusion of the desired gRNA to the U6 promoter, and a second plasmid containing the homology arms and an N- or C-terminal tag. The two PCR reactions are then mixed and transfected along with a Cas9 expression vector. They can also be introduced directly into a *Drosophila* S2 cell line stably expressing Cas9. This PCR toolkit offers C- and N-terminal tagging vectors (eg GFP, Flag, YFP, Strep, TEV-V5) with either blasticidin or puromycin selection.

- Researchers at the Allen Institute for Cell Science recently developed a collection of plasmids to fluorescently tag markers of cellular structures in mammalian cells. These plasmids use fluorescent proteins flanked by long regions of homology to the gene of interest to promote homology directed repair after a CRISPR/Cas9 induced break. Learn more about these constructs and the cell lines they've been used to create in the Allen Institute's recent blog post.

- For more information on other CRISPR/Cas systems used for endogenous protein tagging, please visit our website CRISPR/Cas Plasmids - Protein Tagging.

Beyond simply monitoring protein localization and activity, CRISPR tagging systems can be modified to assay additional protein characteristics. For example, Masato Kanemaki's lab has developed a CRISPR/Cas-based system for tagging endogenous proteins with an auxin-inducible degron (AID) tag to generate proteins which can be rapidly and reversibly degraded after the addition of auxin to the culture medium. Any number of regulatory sequences, including protein enhancers and repressors, can be included in the repair template for inducible alteration of protein expression levels.

CRISPR tagging systems offer users an easy and efficient way to tag endogenous proteins for studying protein expression, localization, and protein-protein interactions. Additionally, thanks to the modular arrangement of vectors included in tagging systems like SapTrap, scientists will be able to generate genome-wide libraries containing fluorescently-tagged proteins at endogenous loci, shedding light on previously unknown protein functions.

Further Reading

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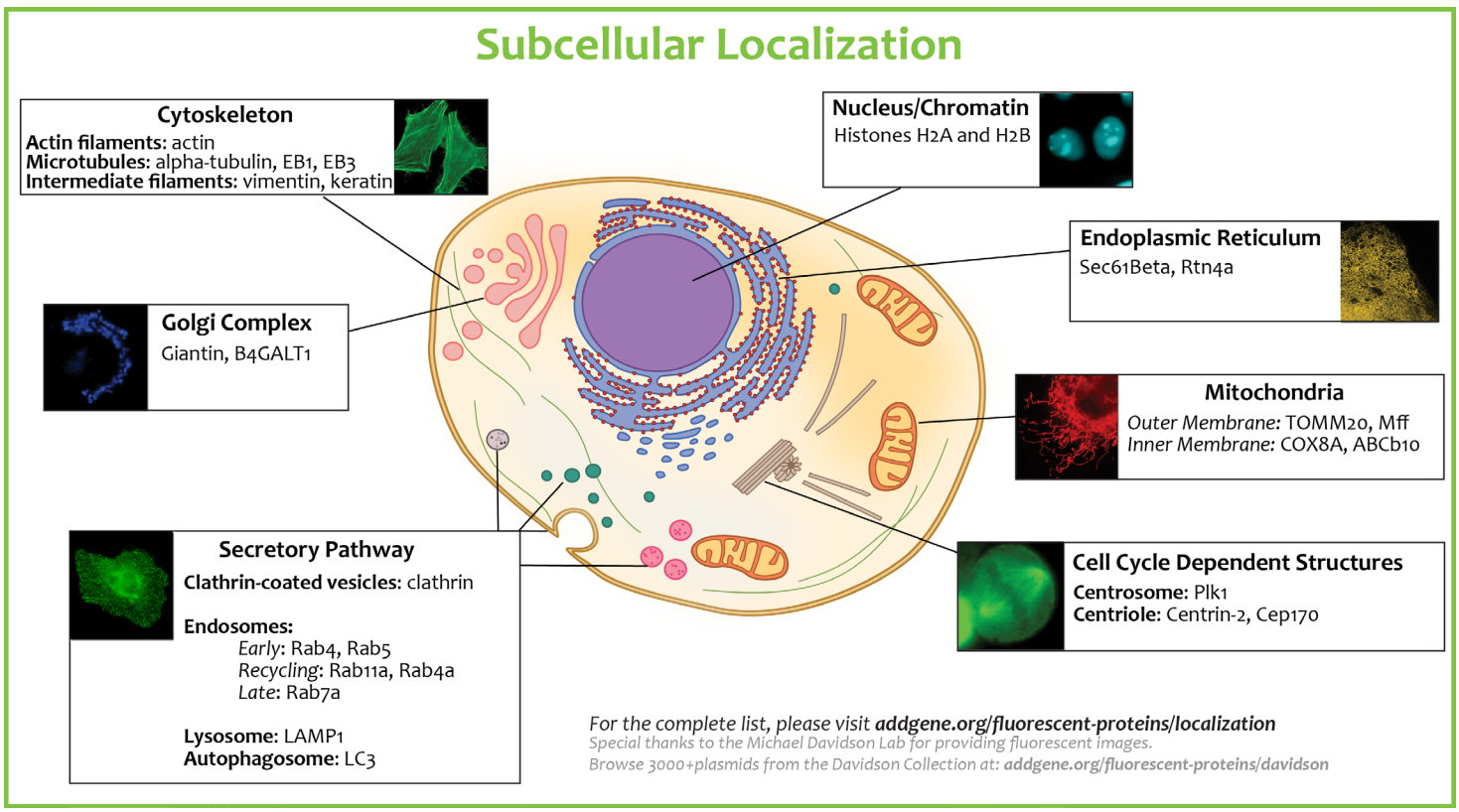
FLUORESCENT TAGGING OF ENDOGENOUS GENES WITH SAPTRAP (CONT'D)

4. Kunzelmann, Stefan, et al. "A Comprehensive Toolbox for Genome Editing in Cultured Drosophila melanogaster Cells." G3 (Bethesda) 6(6) (2016):1777-85. PubMed PMID: 27172193. PubMed Central PMCID: PMC4889673.

CHAPTER 3: USING FLUORESCENT PROTEINS FOR LOCALIZATION

VISUALIZING SUBCELLULAR STRUCTURES & ORGANELLES

By Susanna Bachle | June 22, 2017



The same way the human body is made up of organs, cells comprise compartments and structures, called organelles. Take a sneak peak inside a cell with the images from the Allen Cell Explorer (1).

When studying the function of a protein or its role in a disease, researchers often isolate proteins of interest and examine them using biochemical methods thus removing the context of the cell. However, much knowledge about functionality can be gained by understanding the location and transport of the protein within a living cell. Analyzing differences in protein localization and transport between healthy and diseased states can also provide interesting insights into disease mechanisms and protein function.

Geting Ready to Determine the Subcellular Localization of Your Protein

The first step in analyzing the localization of your protein of interest is to make it detectable. Visualization can be achieved using fluorescent microscopy - even in living cells and whole organisms. You can take advantage of the many Fluorescent Proteins (FP) available for microscopy, by cloning your protein of interest into a vector encoding a fluorescent tag. Once expressed as a fluorescent protein fusion, it is possible to track your protein of interest from its production site to its final destination. As an example, most secreted proteins are produced in the endoplasmic reticulum, modified in the golgi, and then transported in vesicles via the secretory pathway to their subcellular or extracellular destination. At the end of their life cycle, proteins may be transported through the vesicles to the lysosome where they are finally degraded (2, 3).

Once you've created your fusion protein, it's important to validate its function relative to the untagged, wild-type protein. You may, for example, validate that the fusion protein localizes properly by comparing the signal

VISUALIZING SUBCELLULAR STRUCTURES & ORGANELLES (CONT'D)

from the fusion itself to the wild-type protein visualized with a fluorescently-tagged antibody.

By employing "marker proteins" which are known to be a part of an organelle or involved in intracellular transport pathways, it is possible to map the internal organization of a cell. This mapping can be done by using antibodies targeted to the marker proteins, however, in this case the cell needs to be fixed and the cell membrane permeabilized in order for the antibodies to reach their intracellular targets. Therefore, it can be beneficial to employ well-characterized marker proteins tagged with FPs to highlight various subcellular structures. Please see the figure above for commonly used markers for major mammalian organelles and transport pathways.

It is best, though not always possible, to use inert fluorescent protein fusions to visualize subcellular structures. For example, you might use your fluoresent protein fused to a signal sequence that traffics the fluorescent protein to your organelle of interest.

For subcellular markers in yeast see our plasmid collection from Sue Jaspersen (Stowers Institute) and Mark Prescott (Monash University).

You can find more plasmids for labeling your subcellular structure of interest in the Allen Institute for Cell Science Plasmid Collection. You can also use the library of full-length zebrafish rab proteins from Rob Parton's Lab (University of Queensland) to observe membrane trafficking events *in vivo*.

Locate your protein and see who it is hanging out with via colocalization - aka "are they close?"

After tagging your protein of interest and selected subcellular marker proteins, it's possible to get an idea about the subcellular structures your protein resides in and potentially which other proteins it forms complexes with. These "colocalization" studies give insights about the proximity of two proteins within the same subcellular structure or protein complex. By coexpressing both the marker protein and the protein of interest and then analyzing the relative colocalization of their fluorescent signals and the potential overlap, it is possible to determine the location of proteins within complex structures.

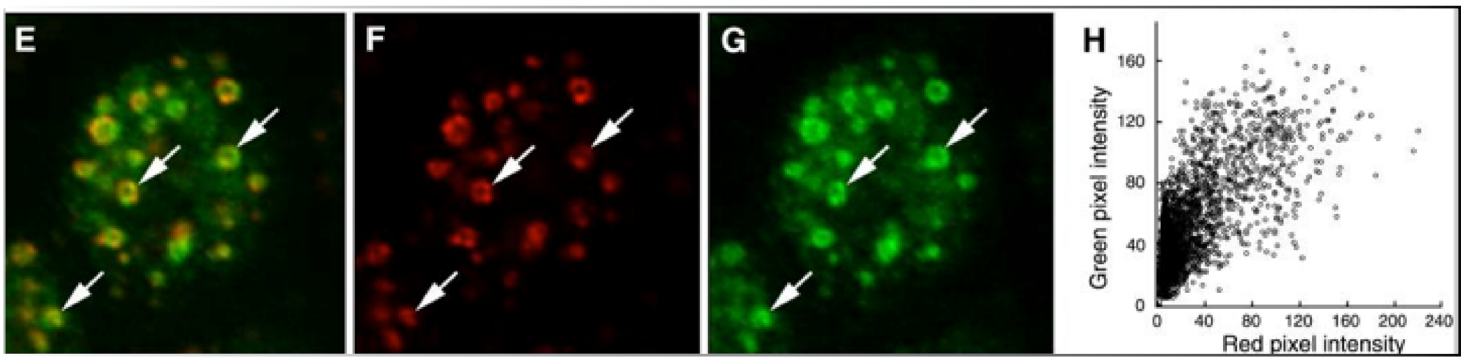
In order to interpret colocalization experiments in a scientific and meaningful way it is important to choose the appropriate quantification methods and tools. It is usually not enough to examine your images using subjective judgement by simply "looking at them". Modern cameras and image analysis software are highly sensitive to detecting light signals not visible to the human eye and are indifferent to subjective color intensity perception. Open source image analysis software tools can be used for colocalization analysis. Some examples include the ImageJ plugins JACoP and Coloc 2, BioImageXD, and customized CellProfiler pipelines. Image analysis software produces quantifiable results and statistics that can be used to compare between different experimental setups.

A colocalization analysis example is given in Figure 2 (adapted from 4) - Dunn et al. 2011 provides an in-depth review of quantitative colocalization analysis methods and useful image analysis software tools.

Pitfalls, Limitations & Specialized Colocalization Methods

Fluorescent proteins are very useful tools, but they are not without limitations and well-executed experiments

VISUALIZING SUBCELLULAR STRUCTURES & ORGANELLES (CONT'D)



Example of a colocalization experiment using red and green FPs. From left to right: Superimposed signals from the red and green channel and the red and green channel alone, respectively. Some compartments labelled with both FPs are highlighted (arrows). The scatterplot demonstrates one analysis method. Here, the red and green intensities of a given pixel are plotted. If the red and green FPs colocalize, you'd expect the two pixel intensities to be correlated and you could fit a straight line to the data (the red signal would be high when the green was high). If the two proteins do not colocalize, you'd expect a more random spread of color intensities to which you couldn't easily fit a straight line . The slope of the scatterplot represents the ratio of the two FP signals. In this example, the points cluster around a straight line, indicating colocalization of the FP signals in the analysed pixels. Adapted from Dunn et al 2011.

require some consideration of FP properties. It is important to look out for general problems associated with fluorescent imaging such as bleed-through (the incorrect detection of one fluorescent signal in a channel setup for a different fluorescent signal) and photobleaching i.e. FP stability.

One should also be aware of additional problems related to the properties of FPs themselves. These can include the potential to form oligomers, protein charge, and actual molecular size – it is possible that a large or charged FP fusion can change the subcellular localization of a protein of interest. Scientists are continuously updating and optimizing FP tools to avoid these problems. See, for example, the recently developed FPs optimized for diverse cellular environments.

Since colocalization relies upon the detection of 2 independent fluorescent signals and their potential overlap, it is essential to make sure that the selected FPs do not influence each other and thus potentially falsify the signal. As a baseline rule, the emission spectra of the selected FPs need to be sufficiently separated, most commonly FPs with red and green wavelengths, respectively, are selected (6). These previous sections help with the decision of which FP you should use and how to select FPs for muli-color imaging.

Furthermore, one must bear in mind that the resolution of light microscopy is limited by the wavelength of light and, practically speaking, for common lab microscopes the detection limit is 200 nm. The size of most proteins is below 10 nm and therefore colocalization detected in microscopy cannot be interpreted as direct molecular interaction without further investigation. One fluorescent microscopy method commonly employed for analyzing interactions between two molecules more closely is Förster Resonance Energy Transfer, FRET.

Additional Opportunities: Tracking of Intracellular Pathogens

In addition to tracking the location of proteins it is also possible to follow the life cycle of viruses and intracellular bacteria – have a look at our Microbiology resources for fluorescently tagged viral and bacterial components. For example, the Rainbow Vectors from the Mariette Barbier Lab can be used to fluorescently label a wide variety of gram negative bacteria (West Virginia University School of Medicine, 7).

Identifying the subcellular localization and composition of cellular complexes are important steps in

VISUALIZING SUBCELLULAR STRUCTURES & ORGANELLES (CONT'D)

understanding the function of your protein of interest in a healthy or diseased cell. We hope you find the resources discussed in this article useful for your next localization experiment and invite you to make any fluorescent protein constructs you create in your own work available to the Addgene community.

Email us at blog@addgene.org to get a full sized version of our Markers of Subcellular Localization Poster.

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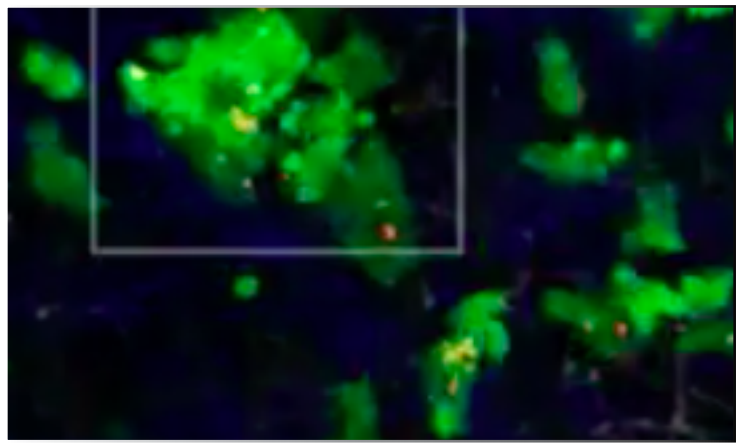
MONITORING CELL MOBILITY USING FLUORESCENT PROTEINS

By Benoit Giquel | May 11, 2017



In complex metazoans, rapid cell division and large scale cell mobility are essential processes during embryonic development. These are required for a growing organism to make the complicated transition from a clump of cells to a fully differentiated body. In contrast, these dynamic processes are largely absent in adult organisms, where tissues structures are more stable and local movements predominate (e.g. a basal progenitor cell migrating to the epithelium). At this stage, only cells from the immune system show wide scale mobility with movement from the bone marrow and other lymphoid organs to specific tissues where they can scan for any signs of danger. In this post we'll focus on how fluorescent proteins can and have been used to monitor cellular movements in the immune system. The techniques used here could be adapted to studying other systems in which there is large scale cellular movement throughout an organism.

Studying cell mobility within these complex environments has never been easy and, for many years, researchers lacked good tools to directly follow immune cells in lymphoid organs. The development of cell sorters together with the engineering of fluorescently labelled antibodies made it possible to track cells from one organ to another. Thus several labs were able to decipher the journey of lymphocytes from the bone marrow to the thymus where they gain their specificity or from the thymus to the lymph nodes. These methods gave researchers much more information regarding immune cell mobility than the static microscopy images of chemically coloured lymphoid organs did in the past.

How Do Researchers Track Fluorescently Labeled Cells?

1. **Epifluorescence microscopy** - This technique uses a fluorescence microscope where the light source is mounted above (epi) the specimen and the excitation light passes through the microscope objective lens on its way toward the specimen. It allows the visualization of fluorescent proteins expressed in the specimen.

2. **Fluorescence Activated Cell Sorter (FACs)** - A machine that sorts cells according to whether or not they have been tagged with a fluorescent protein or dye. It separates the cells mechanically in a vibrating nozzle, imparting a positive or negative charge to cells that fluoresce, and then passing the cells through an electric field to deflect them into appropriate containers. The machine is useful for distinguishing cell populations that have been tagged with different fluorescent proteins or dyes.

3. **Two-Photon Microscopy** - Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissue up to about one millimeter in depth. Two low-energy photons (typically from the same laser) cooperate to cause a higher-energy electronic transition in a fluorescent molecule (see Figure 2 below). The excitation generated by these two-photons occurs only at a chosen focal volume and thus the microscope captures only fluorescence coming from this volume. The consequence is that you can detect signal in thick specimens. See the video below for an example of two-photon intravital microscopy from PLoS Pathogens - Kamenyeva et al, 2015.

MONITORING CELL MOBILITY USING FLUORESCENT PROTEINS (CONT'D)

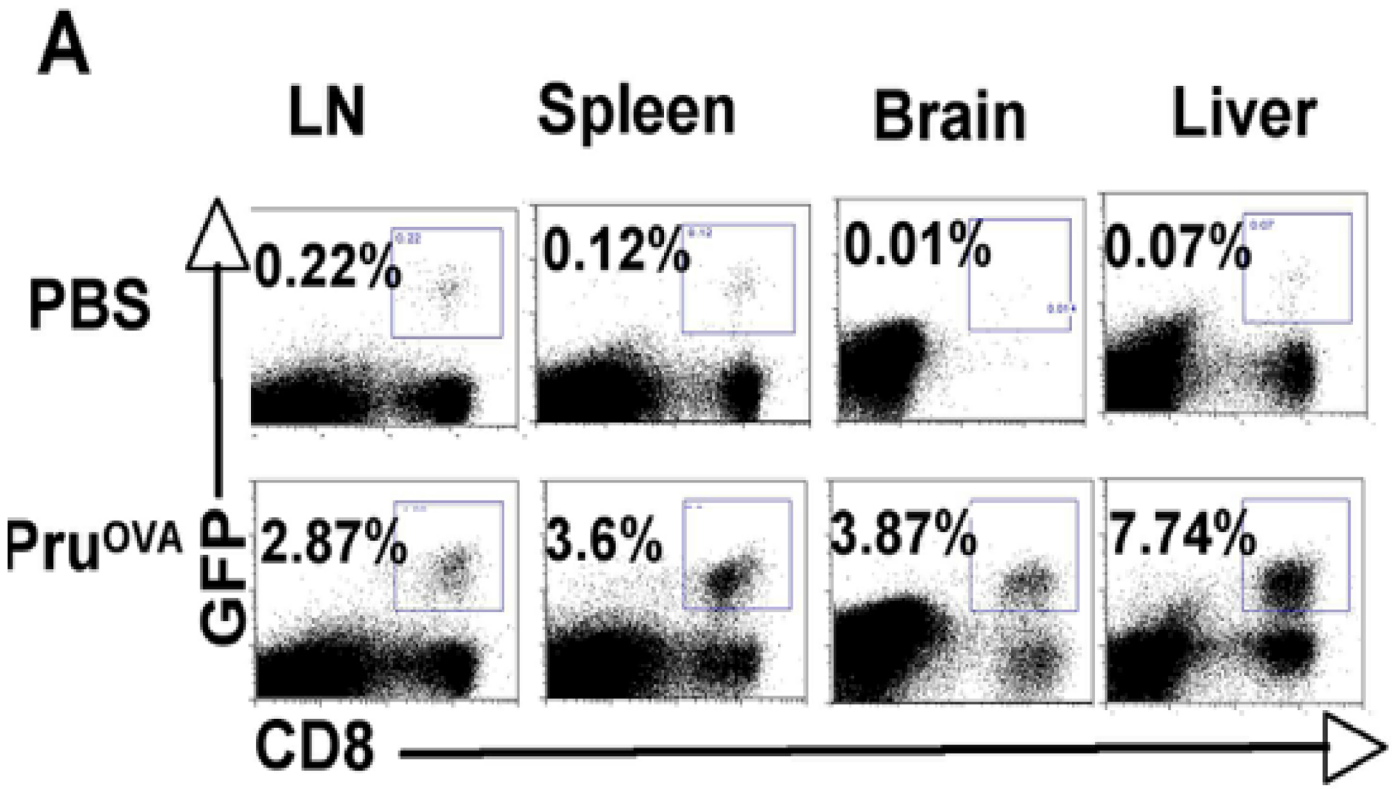


Figure 1: FACS analysis showing the recruitment of GFP-expressing T cells to several organs where a toxoplasma infection occurs. After 7 days of infection with Toxoplasma expressing OVA (PruOVA), mice were injected with GFP-expressing T cells that recognise the OVA antigen. Organs were then harvested and T cells separated by FACS using the indicated GFP and CD8 marker markers (Y and X axes respectively). The results show that OVA-specific T cells (GFP+/CD8+, blue boxes) are recruited to organs that are infected by Toxoplasma. Image from PLoS pathogens John B et al, 2009 https://doi.org/10.1371/journal.ppat.1000505.

Cellular Trafficking Studies Enabled by Fluorescent Proteins

The discovery of GFP and its derivatives by the late Roger Tsien changed everything. For the first time, fluorescent proteins gave researchers the ability to track immune cells within organs and visualize how cells interact after a particular stimulus. Using an epifluorescence microscope and GFP fusion proteins controlled by promoters specific to the immune lineage, scientists could easily track GFP expressing cells. For example, by harvesting lymph nodes at different times after antigen challenge, scientists were able to track where GFP labelled B cells were in mounted slices of these organs. These GFP labelled cells could also be tracked by cell sorter giving immunologists the ability to count cells and statistically analyze cell movements. By using cells labelled by fluorescent chemicals or by other fluorescent proteins, labs were also able to see movement and interaction between several different immune cells. These methods allowed immunologists to develop a spatiotemporal view of an immune response, but the study of these dynamics was still laborious as epifluorescence microscopy wasn't able to give a clear view of cellular movement in real time.

Two-photon microscopy was the next revolution in cell imaging, giving researchers the ability to monitor cellular movements in real time. This technique was first Introduced to immunology by three papers in the journal Science in 2002. Now, this technique and the *in vivo* live-cell imaging (intravital imaging) studies it's enabled are revealing the cellular behaviors that mediate adaptive and innate immunity in diverse tissue environments. These studies provide quantitative measurements of cellular motility, interactions, and response dynamics. In the past 15 years efforts have been made to create transgenic mice expressing fluorescent protein reporter

MONITORING CELL MOBILITY USING FLUORESCENT PROTEINS (CONT'D)

constructs in the immune lineage. By specifically labeling T cells, B cells, and antigen presenting cells, scientists have been able to decypher cellular dynamics in lymphoid organs. In lymph nodes, for example, two-photon microscopy allowed scientists to better understand T cell initiation in the adaptive immune response. It was known that T cells were able to extravasate from the bloodstream to invade lymph nodes and scan antigen presenting cells, but two-photon microscopy enabled scientists to see how chaotic and random the scanning was and how fast T cells could jump from one antigen presenting cell to another. Indeed, we now know that T cells are able to crawl more rapidly than any other cell type in the body!

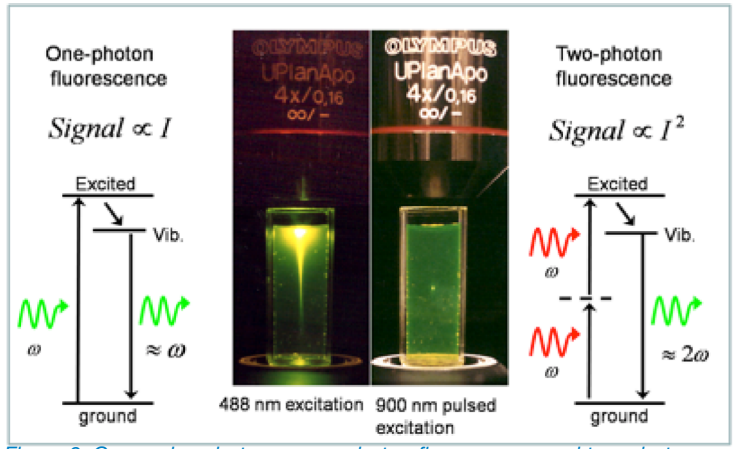


Figure 2: Comparison between one photon fluorescence and two photon fluorescence. Left: One photon fluorescence in a fluoroscein solution. One photon fluorescence uses a blue scanning laser that excites an entire column of sample. Right: two-photon fluorescence in the same solution. Two photon fluorescence uses a pulsed infrared (IR) laser that excites only a small spot of sample. If two photons simultaneously interact with a fluorophore at a wavelength approximately twice the excitation maximum of the fluorophore, it can be excited. These events generally are restricted to the exact focal point of the laser, so an extremely small volume is excited. Image from Webb Lab, Cornell University, Adapted by J. van Howe.

Intravital imaging powered by two-photon microscopy has uncovered host-pathogen interactions leading to the understanding of effector function in infected tissues. Before the use of two-photon imaging, our understanding of pathogen-immune cell interaction relied on *in vitro* studies where it was difficult to apprehend the key role of specialized cell types and organs that exist *in vivo*. The use of fluorescent pathogens and fluorescent cells enables scientists to monitor cell interactions in diverse tissues, including lymph nodes, brain, liver, gut, and skin.

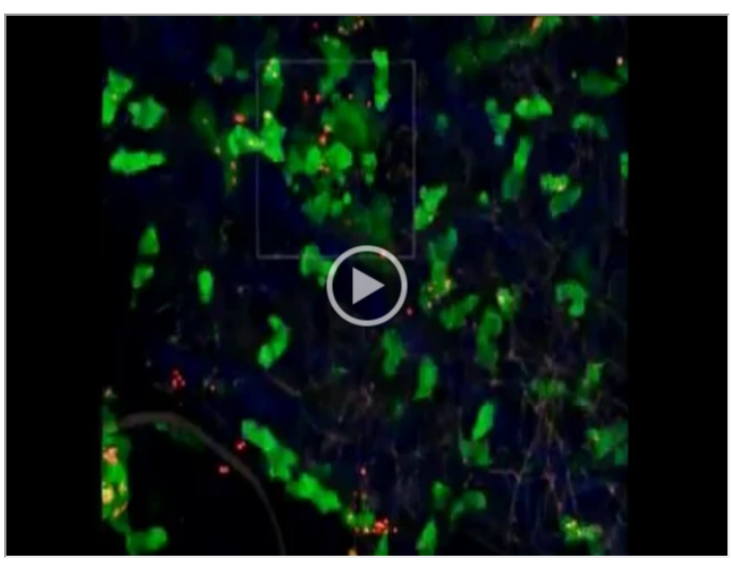


Figure 3: Two-photon intravital microscopy. Check out the video on our associated blog post showing the infiltration of neutrophils expressing LysM-GFP (green) into a draining lymph node after infection with S. aureus *(red). You can then see neutrophils swarming around* S. aureus *and eating the bacteria.*

Some of the many questions scientists have been able to help answer using two-photon microscopy include:

- How do T cells and/or Neutrophils travel to the sites of infection or damage (Peters et al. 2008, Chtanova et al. 2008, Kamenyeva et al. 2015)?
- How do blood monocytes patrol blood vessels during immune responses (Auffray et al. 2007, Finsterbusch et al. 2016)?
- How do dendritic cells migrate to lymph nodes to activate T cells (Celli et al. 2008, Kitano et al. 2016, Cavanagh et al. 2008)?

Technical Challenges of Two Photon Microscopy

Intravital imaging is an easy way to understand what is going on in the body at steady state or under physiopathological challenge. However technical specificities have to be taken into consideration especially in the use of fluorescent proteins to track cellular movements and interactions. Even if virtually all fluorescent proteins can be used in your experiments, experience has shown that some are more reliable and more efficient at tracking cells. Good two-photon probes when using a

MONITORING CELL MOBILITY USING FLUORESCENT PROTEINS (CONT'D)

standard Ti:Sapphire Laser include:

- Blue-green fluorescent proteins like EGFP or mTFP
- Yellow-orange fluorescent proteins like TagRFP, tdTomato, DsRed, the mKate series, or tdKatushka2 (Drobizhev et al. 2011)

The commonly used orange and red fluorescent proteins are excited by 750 nm to 760 nm laser light, enabling dual color imaging studies with blue or cyan proteins without changing excitation wavelength (Salomonnson et al. 2012). They can also be excited efficiently at wavelengths between 1,000 nm and 1,200 nm, where there is relatively little tissue absorption, weak tissue scattering, and small amounts of tissue auto-fluorescence (Drobizhev et al. 2011). But this requires other lasers than the standard Ti:Sapphire laser. It is also important to finely tune the excitation wavelength as it has been shown that even a small incremental change of the excitation wavelength can significantly affect emission intensities from fluorescent proteins (Salomonnson et al. 2012).

Additional Fluorescent Protein Tools

Photoactivable fluorescent proteins (proteins that fluoresce after a light-induced chemical reaction) or photoconvertible Keade proteins (protein that undergoes light-induced irreversible photoconversion from green fluorescence to red fluorescence) are also useful tools to use with intravital imaging. These tools allow one to study the kinetics of cell migration and movement by marking cells at a specific time. Photoactivable fluorescent proteins, for example, have been successfully used to study B and T cell dynamics within the germinal center of murine lymph nodes. However, one must pay attention to time scale when using photoactivatable FPs - their degradation within labelled cells limits the timespan available for tracking certain cell types.

Another useful tool for marking and tracking cells is the the Cre/lox recombination system. A researcher can flank fluorescent proteins in a plasmid with loxP sites such that their expression is turned on or off in cells expressing Cre. The result of a system like this is a mosaic of cells labelled with different fluorescent proteins. Using one iteration of this system called Brainbow, the Ubow mouse strain has been created to fate map Langerhans cells and follicular dendritic cells within the skin and lymph node respectively. The permanent labelling of cells using cre-lox is advantageous when compared to photoactivable or photoconvertible proteins, as it enables tracking throughout the lifetime of the cell.

The past 15 years have seen a lot of development surrounding the use of fluorescent proteins and intravital imaging in understanding the key roles of immune cells in triggering an immune response. New fluorescent proteins can be used more efficiently in two-photon microscopy and new constructs have facilitated the creation of mouse lines that can be used in fate mapping experiments. Immune response dynamics are much better understood thanks to these technologies but many questions remain. The use of other technologies such as optogenetics and CRISPR/Cas9 will help immunologists create even better tools and models to further our understanding of the immune system and its dynamics.

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VISUALIZING TRANSLATION AT THE SINGLE MOLECULE LEVEL

By Mary Gearing | March 22, 2017

Regulating translation is key to cellular function, especially during development or stress. With ribosome profiling, researchers have been able to study the effects of various stimuli on global translation, but a visual technique to study translation remained elusive. Two techniques developed by Addgene depositors have made it easier to track translation in two different ways: by monitoring the first round of translation or by tracking the translation of a single mRNA over time. Both are helping researchers explore the complexity of translational control in cellular physiology.

Ribosome Profiling

Ribosome profiling, a biochemical method developed in 2009, takes a "snapshot" of all of the mRNA bound-ribosomes in a cell. When cellular mRNAs are digested with ribonucleases, ribosomes protect the segments of mRNA to which they are bound. These regions are subsequently sequenced and aligned to the genome to determine genome-wide translational frequency. Although highly useful, this technique requires extensive sample processing to avoid disrupting ribosome-mRNA complexes. Recent work has adapted this technique to monitor local translation and determine if proteins are targeted to their destination co- or post-translationally.

TRICK: Visualizing the First Round of Translation

While ribosome profiling co-opts the physical location of the ribosome, TRICK (Translating RNA Imaging by Coat protein Knock-off) instead takes advantage of the ribosome's movement. When the ribosome moves along an mRNA, it displaces other RNA-binding proteins to allow translation to occur. For a TRICK reporter mRNA this displacement leads to a change in the fluorescent signal: untranslated RNAs appear yellow, and translated RNAs appear red. To enable this color switching, TRICK requires the following components:

1. A GFP protein that binds to the coding region of a reporter mRNA via a PP7 coat protein (NLS-PCP-GFP)
2. An RFP protein that binds to the 3' UTR of the reporter mRNA via an MS2 coat protein (NLS-MS2-RFP)
3. The reporter mRNA, which contains the tethering sites for the fluorescent proteins and is driven by an inducible promoter (Plasmid 64542 or 64543)

Before translation, both GFP and RFP are bound to the reporter, causing it to appear yellow. The act of translation pushes NLS-PCP-GFP off of the mRNA, leaving only a red FP bound to the reporter. Because it contains a nuclear localization signal, NLS-PCP-GFP then returns to the nucleus to bind cognate untranslated mRNAs. The high specificity and resolution of this system are achieved by using multiple copies of the PP7 and MS2 coat protein binding sites to tether many copies of the FP-coat protein fusions to their reporter mRNAs. Using translation inhibitors cycloheximide and puromycin, Halstead et al. verified that translation is necessary for removal of GFP from the TRICK reporter.

Delayed translation is known to be important in development, and Halstead et al. used TRICK to examine translation of *Drosophila* patterning gene oskar. Early in development, the osk-TRICK reporter was double labeled, indicating translational repression. During later stages, single RFP-labeled osk and Oskar protein were detected at the posterior pole. Oskar protein levels were negatively correlated with GFP intensity, showing that TRICK is an accurate readout of relative reporter translation.

VISUALIZING TRANSLATION AT THE SINGLE MOLECULE LEVEL (CONT'D)

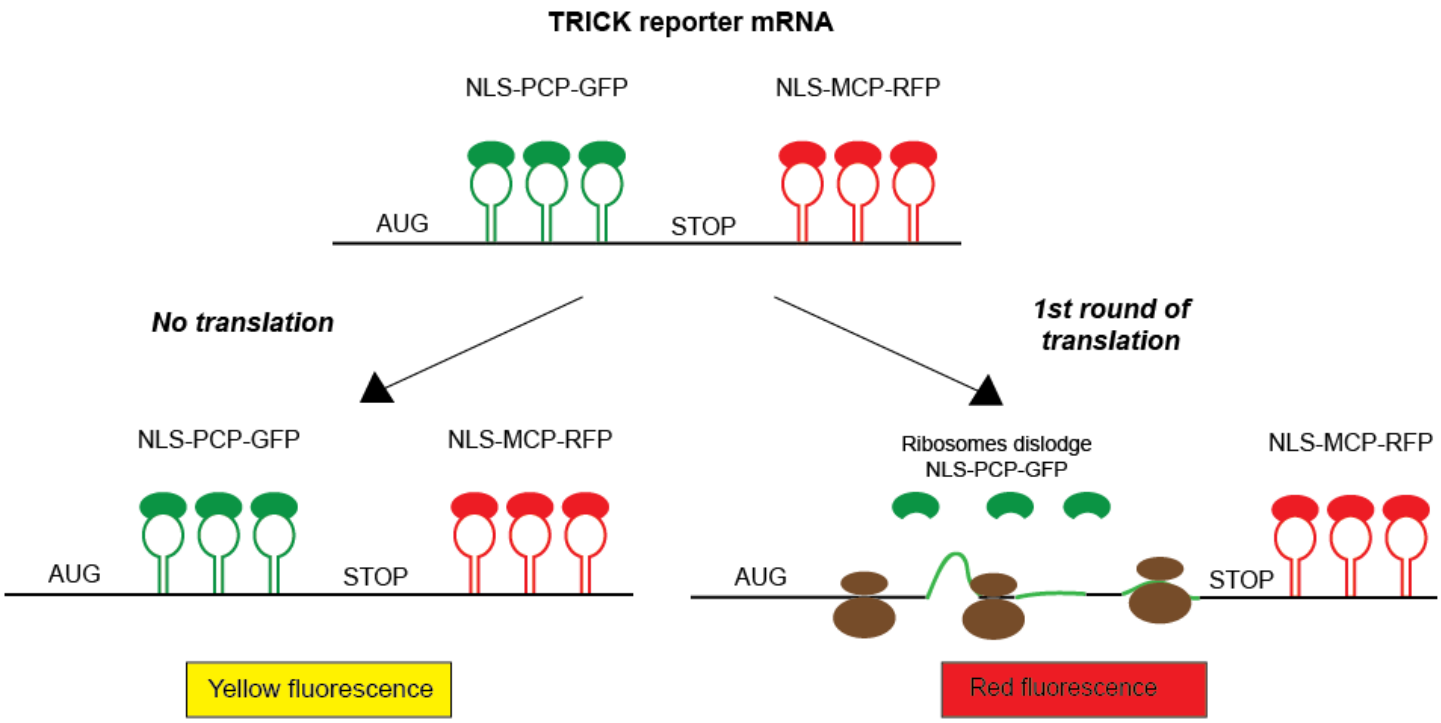

Figure 1: The TRICK reporter mRNA contains two types of hairpins. Hairpins in the coding sequences bind NLS-PCP-GFP, while hairpins in the 3' UTR bind NLS-mCP-RFP. Untranslated RNAs display yellow fluorescence. During the first round of translation, ribosomal movement dislodges NLS-PCP-GFP, converting mRNA fluorescence to red.

Single Molecule Continuous Translation Tracking

TRICK allows you to see the first round of translation, but it doesn't allow continued tracking of a given mRNA because NLS-PCP-GFP is removed by the first ribosome that translates the mRNA. To permit tracking over time, Yan et al. devised a system that labels both mRNAs and their corresponding newly synthesized proteins. As in the TRICK system, the 3' UTR of the reporter mRNA is labeled by PCP-mCherry (Figure 2). The 3' UTR also contains a CAAX sequence to bind the mRNA to the plasma membrane; this sequence prevents diffusion of the mCherry labeled mRNA and keeps it in a single field of view for constant tracking. You can then directly monitor multiple rounds of translation from this single mRNA; you get a view of translational dynamics at the single molecule level.

The coding sequence of Yan et al.'s reporter contains 24 copies of SunTag, a synthetic scaffold that can recruit GFP fused to the SunTag-specific antibody scFv. Each time the reporter is translated, the SunTag sites on the nascent polypeptide recruit scFV-GFP, generating green puncta that colocalize with the red reporter mRNA. Yan et al. used harringtonine, a translation inhibitor that stalls ribosomes after initiation, to calculate a ribosome translocation rate of ~3.5 codons/second. They also explored the effects of 5' UTR variants in translation of cell cycle protein Emi1, showing that ~80% of transcripts with a long 5' UTR were translationally silent.

Both of these methods increase our understanding of translation dynamics, but they come with a few caveats. In both cases, creating a reporter mRNA requires inserting multiple hairpin sequences into the coding sequence and 3' UTR. Before beginning experiments, it's important to verify that these changes do not impact translation rate, as the authors of these papers did. Since the SunTag-based system tethers mRNAs to the plasma membrane, it's not appropriate for use with mRNAs usually translated in a specific compartment. Despite

VISUALIZING TRANSLATION AT THE SINGLE MOLECULE LEVEL (CONT'D)

these limitations, these techniques represent powerful tools for studying translation in various cell types and physiological states.

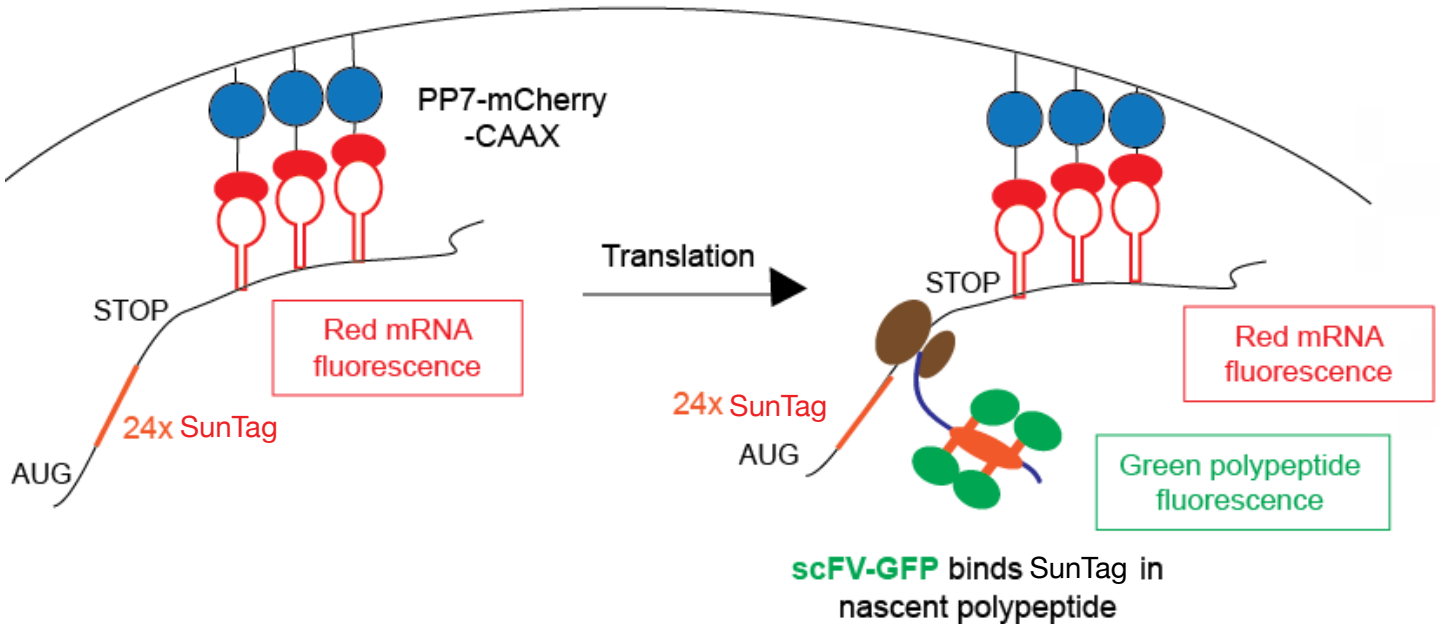

Figure 2: Tracking translation over time. The reporter mRNA contains a 24x SunTag scaffold in the coding region and hairpins in the 3' UTR that bind PP7-mCherry-CAAX, which tethers the reporter to the membrane. When translation occurs, the SunTag sequences on the nascent polypeptide bind scFV-GFP, creating green puncta near the red mRNA.

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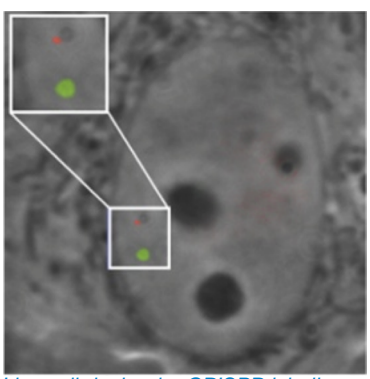
MAPPING THE 4D NUCLEOME WITH CRISPR/CAS9

By Mary Gearing | August 11, 2015

It seems that there's a new CRISPR advance or technique published every week! One of these applications is a colorful system that uses fluorescently labeled Cas9 to label multiple genomic loci in live cells. While other systems can be used to label loci, such as fluorescence in situ hybridization (FISH) or fluorescently labeled TALEs, CRISPR/Cas9's ease of use and ability to label live cells make this system truly advantageous. This new technique, developed in Thoru Pederson's lab, brings us one step closer to mapping the 4D nucleome, the organization of the nucleus in space and time, and to understanding how nuclear organization varies across the life of a cell, or how organization may be altered in disease states.

dCas9 Gets a Colorful Makeover

Even without its nuclease activity, catalytically dead Cas9 (dCas9) has many applications, the most well-known being transcriptional activation/repression. dCas9 has also previously been adapted to fluorescently label a sequence matching its gRNA, facilitating the live study of chromatin dynamics at a particular locus. A limitation of this system is the single-color labeling; multicolor labeling would enable the visualization of multiple loci. Such a system could then be used to examine both inter- and intrachromosomal dynamics, and how they change in response to the cell cycle or other stimuli.



Live cell dual-color CRISPR labeling of two loci on human chromosome 9

To create a colorful Cas9 toolbox, Ma et al. turned to SpCas9 and its orthologs NmCas9 and St1Cas9. Each ortholog was fused to a different fluorescent protein to create three colors. The specificity of these orthologs is key: due to differences in the PAM sequences required by each ortholog, a gRNA designed for one dCas9 should be specific to that ortholog and not cross-talk with the other orthologs.

Ma et al. tested their dCas9 variants using gRNAs specific for telomeric sequences and showed that different fluorescently labeled dCas9 isoforms are efficiently directed to the proper target sequence. They were also able to label two different pairs of chromosomes using gRNAs specific to sequences on chromosomes 9 and 13.

They next turned their attention to mapping pairs of intrachromosomal loci. The technique successfully resolved loci with physical map distances of 75 and 2 Mbp, with the calculated fluorescent distances correlating with the previously established physical map. In comparing pairs of targets ~2 Mbp apart, they noticed that they could evaluate the degree of chromatin compaction even for this small distance! To the authors' knowledge, this work represents the first mapping of intrachromosomal loci, a major benchmark in characterizing the 4D nucleome.

Future Modifications and Applications

Ma et al. developed this technique using standard fluorescence microscopy, and are thus limited by its lower resolution. The combination of this method with superresolution microscopy may improve the resolution, although background signal would be a concern. Another caveat of the study is the method's sensitivity. The authors estimate that a minimum of 150-200 fluorescent protein molecules is necessary for a detectable signal, limiting the sensitivity of the technique. One potential solution is SunTag, a synthetic scaffold that can be used to recruit multiple protein molecules. Since the signal level provided by SunTag is so high, cells may be imaged under lower illumination settings, lowering photobleaching and phototoxicity concerns and extending the potential imaging time for this technique.

MAPPING THE 4D NUCLEOME WITH CRISPR/CAS9 (CONT'D)

The NIH Common Fund has made mapping the 4D nucleome a specific priority, as researchers work to increase our understanding of chromatin organization. This fluorescent CRISPR/Cas9-based method is a great step forward in live cell imaging of genomic loci. Ma et al. anticipate that the technique will be applicable to studies of cell cycle progression, epigenetics and cellular reactions to external stimuli. It could also have major applications to cancer, for example, the visualization of chromosomal translocations or chromosomal shattering (chromothripsis.) As this study joins the pantheon of useful CRISPR/Cas9 techniques, we at Addgene are excited to see what's coming next!

Further Reading

1. Ma, Hanhui, et al. "Multicolor CRISPR labeling of chromosomal loci in human cells." Proceedings of the National Academy of Sciences 112.10 (2015): 3002-3007. PubMed PMID: 25713381. PubMed Central PMCID: PMC4364232.

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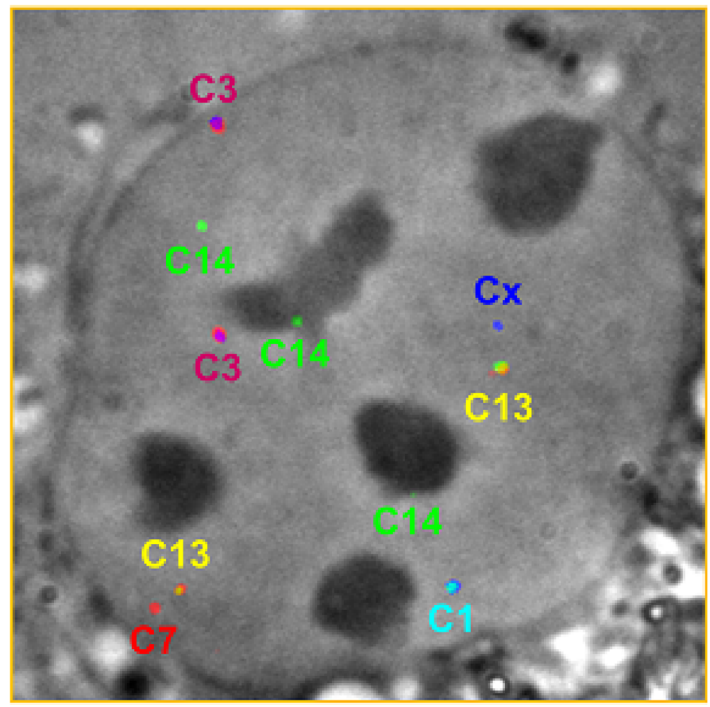
CRISPRAINBOW AND GENOME VISUALIZATION

By Mary Gearing | February 28, 2017

Colorful CRISPR technologies are helping researchers visualize the genome and its organization within the nucleus, also called the 4D nucleome. Visualizing specific loci has historically been difficult, as techniques like fluorescent in situ hybridization (FISH) and chromosome capture suffer from low resolution and can't be used *in vivo*. Some researchers have used fluorescently tagged DNA-binding proteins to label certain loci, but this approach is not scalable for every locus... unlike CRISPR. Early CRISPR labeling techniques allowed researchers to visualize nearly any single genomic locus, and recent advances have allowed scientists to track multiple genomic loci over time using all the colors of the CRISPRainbow.



CRISPRainbow was used to track the localization of 6 chromosome-specific loci in the U2OS cancer cell line. Each color represents a gRNA targeting a specific chromosome. This image represents the composite of fluorescent measurements from red, blue, and green channels overlaid on a bright-field image.

Using dCas9 to Visualize Genomic Loci *In Vivo*

The flexibility of CRISPR has improved our ability to target almost every genomic locus, and to do so *in vivo*. Catalytically dead Cas9 (dCas9) cannot induce gene editing, but it retains its gRNA-directed targeting capacity. By using a gRNA to target fluorescently labeled dCas9 to a given genomic locus, you can track the localization and movement of this locus in live cells! This technique was developed in the lab of Addgene depositor Stanley Qi and used in Chen et al. to track telomere dynamics throughout the cell cycle. To optimize the signal to noise ratio of the system, they modified GFP-dCas9 and the gRNA scaffold to enhance complex assembly, thereby decreasing the amount of background fluorescence from unbound GFP-dCas9. With these improvements, labeling efficiency was similar to that of a comparable FISH method. Importantly, they did not observe labeling when a.) no gRNA was present or b.) when a gRNA binding a non-mammalian sequence (GAL4) was supplied.

In addition to labeling repetitive telomeric sequences, Chen et al. successfully labeled protein-coding genes with both intronic and exonic gRNAs. In fact, the method is specific and sensitive enough to detect gene copy number based on the number of fluorescent puncta observed. By labeling two genes of a given chromosome simultaneously, they could also monitor the spatial relationship of the two genes over time, at a range of distances from 2-75 Mbp.

Labeling Multiple, Intrachromosomal Loci

Building on the work of Chen et al., Thoru Pederson's lab used CRISPR to label multiple loci in distinct colors. To create a colorful Cas9 toolbox, Ma et al. turned to SpCas9 and its orthologs NmCas9 and St1Cas9. Each ortholog was fused to a different fluorescent protein to create three distinct colors. The specificity of these orthologs is key: since each ortholog requires a different PAM sequence, a gRNA designed for one dCas9 should be specific to that ortholog and not cross-talk with the other orthologs.

CRISPRAINBOW AND GENOME VISUALIZATION (CONT'D)

Ma et al. tested their dCas9 variants using gRNAs specific for telomeric sequences and showed that different fluorescently labeled dCas9s are efficiently directed to the proper target sequence. They succeeded in labeling two different pairs of chromosomes using gRNAs specific to sequences on chromosomes 9 and 13. They next turned their attention to mapping pairs of intrachromosomal loci. The technique successfully resolved loci with physical map distances of 2 and 75 Mbp, with the calculated fluorescent distances correlating with the previously established physical map. In comparing pairs of targets ~2 Mbp apart, they noticed that they could evaluate the degree of chromatin compaction even for this small distance! To the authors' knowledge, this work represents the first mapping of intrachromosomal loci, a major benchmark in characterizing the 4D nucleome.

CRISPRainbow: Scaling up to 6 Colors + White

FP Color	Hairpin
Blue	MS2-MS2
Green	PP7-PP7
Red	boxB-boxB
Cyan	MS2-PP7
Yellow	PP7-boxB
Magenta	boxB-MS2
White	boxB-MS2-PP7

Despite the success of Ma et al.'s approach, fluorescently labeled Cas9's come with a number of limitations. Since color is specified by PAM sequence, each color requires a different Cas9 ortholog, and the target sequence must be located adjacent to that PAM. To scale up fluorescent CRISPR labeling, they took a new approach: labeling the gRNA itself.

In their CRISPRainbow paper, Ma et al. engineered gRNA scaffolds containing two hairpin sequences, which recruit fluorescent proteins BFP, GFP, and RFP fused to the respective hairpin binding domain. Pairs of identical hairpin sequences specify the primary colors blue, green, and red. In contrast, a gRNA with two different hairpins will produce a secondary color cyan, yellow, or magenta, depending on the hairpin combination bringing the total number of individual colors to 6. A gRNA with all three hairpins generates white light. All 6 gRNAs can be expressed in a single vector, pCRISPRainbow-DONOR1, with dCas9 supplied by a separate vector.

With this rainbow of colors, Ma et al. performed sophisticated tracking of multiple chromosomal loci in live cells. However, they note that certain improvements could make CRISPRainbow even better. Adding an additional hairpin/fluorescent protein combination, for example, with a far-red FP, would increase the available colors to 15! They also envision using CRISPRainbow in combination with gene editing. CRISPRainbow requires very short, 11-mer gRNAs, which do not induce genome editing. If used with catalytically active Cas9, the short, CRISPRainbow gRNA will permit labeling, but a newly expressed standard length gRNA would "switch" the system and induce genome editing. Such a system has been previously described for transcriptional activation/repression and genome editing.

CRISPRainbow generates 6 unique colors and a white composite. gRNAs with two identical hairpin binding domains produce blue, green, or red fluorescence, depending on the hairpin binding domain sequence (as noted in the table). gRNAs with two different hairpin binding domains produce yellow, cyan, or magenta light. A gRNA with all 3 distinct hairpin binding domains produces white light.

CRISPRAINBOW AND GENOME VISUALIZATION (CONT'D)

Further Reading

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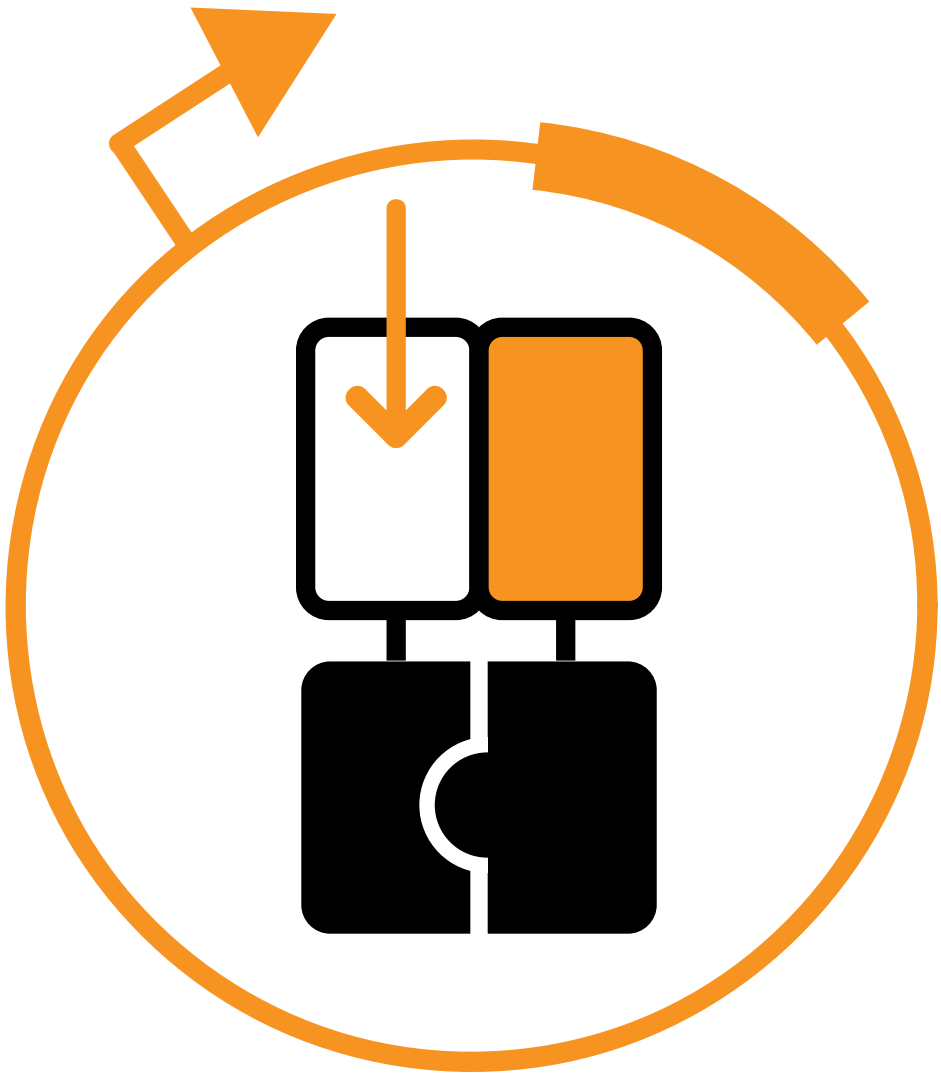
CHAPTER 4: FRET



INTRODUCTION TO FRET

By Jason Niehaus | June 6, 2017

Imagine being able to determine whether two proteins are within 10 nanometers of each other, or measure the tension in the helical structure of spider silk, or the activity of a protein in a synapse. What kinds of tools enable us to measure these properties, and what fascinating experiments could push these tools even further? All of these things can be done using FRET! Read on to find out more about this amazing imaging technique.

What is this FRET You Speak of?

Förster Resonance Energy Transfer (FRET) was originally described by Theodor Förster in 1948 as a variation of the more commonly observed light emission by fluorescence. The widespread use of FRET with fluorescent molecules, including fluorescent proteins, has led to the alternative acronym Fluorescence Resonance Energy Transfer. Unlike the typical excitation and emission of an excited fluorophore, FRET involves a non-radiative transfer of energy (i.e. no emitted photons) from the excited donor fluorophore to the acceptor fluorophore. The typical steps in FRET are:

1. Donor fluorophore excitation by absorption of a photon

2. Energy transfer from the excited donor directly to the acceptor fluorophore--think of it as a virtual photon

3. Relaxation of the acceptor fluorophore back to its ground state by emission of a photon with wavelength specific to the acceptor

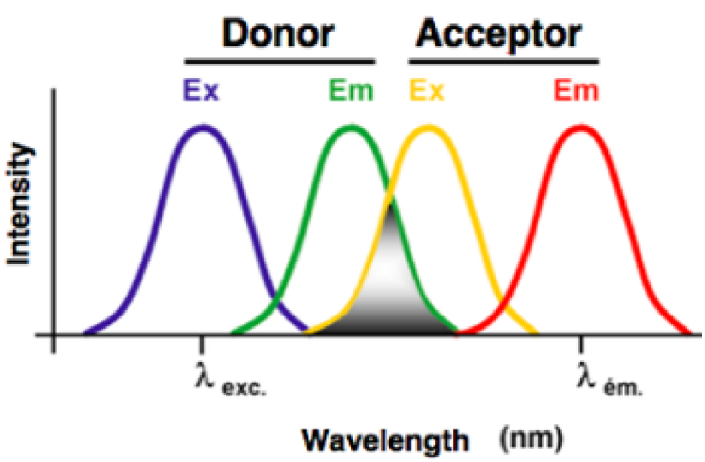


Figure 1: Spectral overlap required for FRET to occur. Modified from Wikipedia, original author Maurel Damien. Accessed 11/4/2014.

In order for FRET to occur several constraints must be met. The donor and acceptor fluorophores must be compatible, such that the emission spectrum of the donor fluorophore overlaps with the excitation spectrum of the acceptor fluorophore. Otherwise the energy transferred from the donor will not be able to excite the acceptor. In addition to sufficient spectral overlap, the fluorophores must be located within 1-10 nm of each other and be oriented appropriately for energy transfer via dipole-dipole interaction.

The efficiency of FRET can be measured for a given donor-acceptor pair and a change in FRET efficiency correlates with a change in the distance and/or orientation of the FRET pair. Since many biological processes occur within the typical FRET range, FRET efficiency is used to infer an interaction between the fluorophores and serves as a small-scale ruler to measure distances that are too miniscule for conventional light microscopy.

Don't FRET - Use These FRET Resources Instead!

As the choice of fluorophore pairs is a key consideration for a FRET experiment, the possibilities can seem overwhelming given the large assortment of fluorescent proteins available. Fortunately, several excellent reviews have collated data from various papers to make the decision easier.

INTRODUCTION TO FRET (CONT'D)

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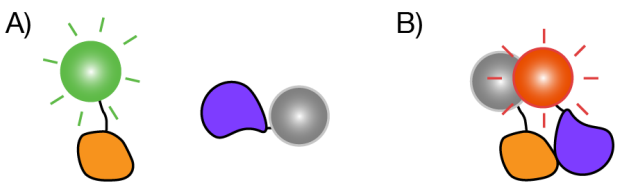

Figure 2: Intermolecular FRET. A) When two fluorescent protein fusions (orange and purple) don't interact, there is no transfer of energy from the green fluorophore to the red fluorophore and so the red fluorophore is dark (gray here). B) When the two fluorescent protein fusions do interact, non-radiative energy transfer from the green fluorophore to the red fluorophore causes the red fluorophore to fluoresce instead of the green fluorophore.

Many empty vectors containing these fluorescent proteins are available on our FRET Resource page and can be used to create fusions with a gene of interest. These plasmids will be most useful for constructing intermolecular FRET probes, where the donor and acceptor fluorophores are fused to two separate proteins. (In contrast, intramolecular FRET probes contain the donor and acceptor fluorophore on the same protein and are useful when a process affects the conformation of the probe. More on these biosensors later on). Experiments using intermolecular FRET probes are typically used for studying protein-protein interactions by measuring the change in FRET efficiency, from which conclusions regarding the proximity of the two proteins can be inferred.

Intermolecular FRET can be experimentally difficult to achieve, because the ratio of acceptor to donor fusions varies with transfection efficiency and any unpaired fluorescent proteins can contribute additional noise to the measurement. If the distance or orientation of the donor and acceptor proteins is not optimal, FRET may not occur or be detected even if the two proteins form a complex.

To help troubleshoot your experimental setup as a potential source of error in suboptimal or undetectable FRET, well-characterized FRET reference standards can be used to validate FRET measurements and serve as a type of positive control. In the case of an unfavorable donor-acceptor orientation limiting FRET efficiency, circular permutation (Baird et al., 1999) of the fluorescent protein (i.e. rearranging the start and end positions without changing the order of the amino acids in the protein) may be able to boost FRET efficiency.

Before constructing your own FRET probe, try searching PubMed for articles describing the FRET tool

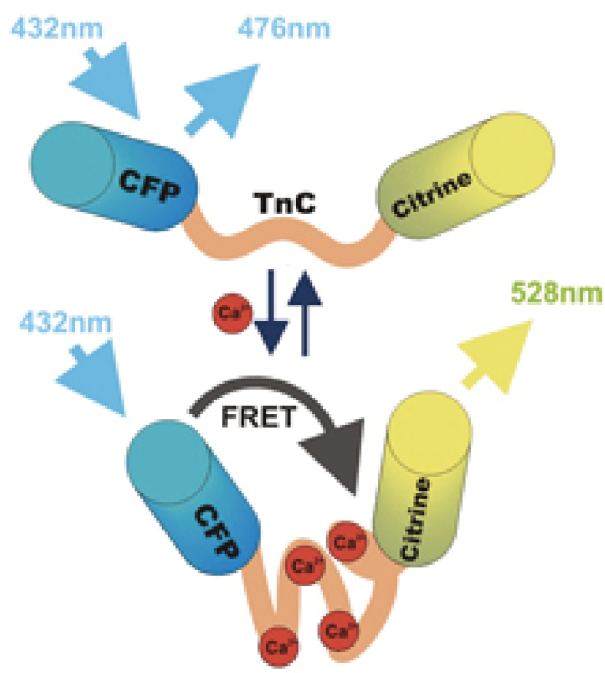

Figure 3: An example of intramolecular FRET in a FRET-based biosensor. Here, calcium binding causes a change in the fusion protein structure that brings the CFP and Citrine components in close enough proximity to transfer energy from CFP to citrine. Citrine subsequently fluoresces. Image credit: MPI of Neurobiology/Griesbeck.

INTRODUCTION TO FRET (CONT'D)

that you are looking for and check our curated list of biosensors too, as another laboratory may have already created the sensor that you need. FRET biosensors designed to measure specific small biomolecules or gene activity are often intramolecular probes, as the linker sequence between the donor and acceptor is sensitive to a change in the environment which alters the FRET efficiency. Detecting certain cellular changes with an intermolecular probe is often either impractical (for biomolecules that are not proteins) or would perturb the endogenous state that you want to measure (overexpressing a gene or protein after transfection).

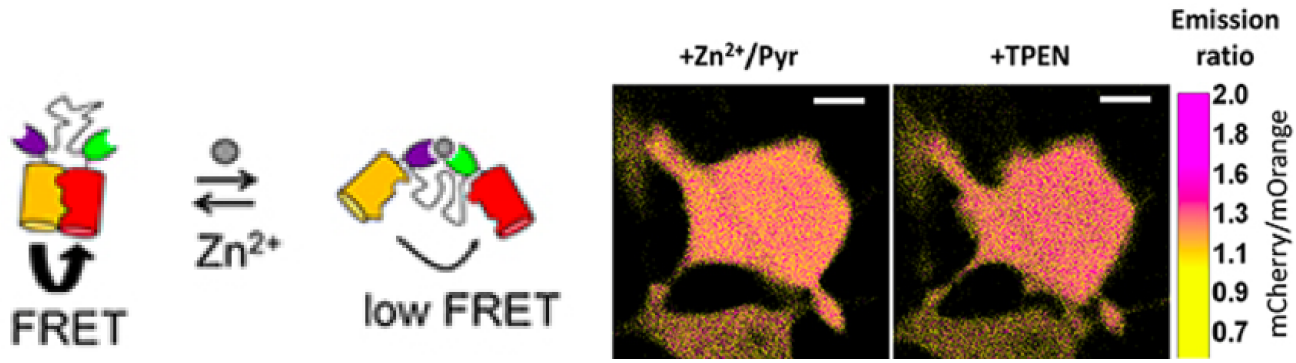

Figure 4: A FRET based biosensor for zinc. In this biosensor, addtion of zinc decreases the FRET signal. In the absence of the zinc, mOrange transfers energy to mCherry resulting in a high mCherry/mOrange emission ratio (+TPEN). in the presence of zinc, low FRET decreases the mCherry/mOrange emission ratio (+Zn^{2+}/Pyr). Image adapted from Lindenburg et al 2014.

Future of FRET

The first genetically encoded FRET biosensor, Cameleon (Miyawaki et al., 1997), was designed to measure intracellular calcium and published in 1997. Since that time, numerous advances in probe design, fluorescent proteins and microscopy equipment have enhanced the ability of labs to answer sophisticated questions about cellular processes. Currently, FRET experiments can probe protein-protein interactions, measure the concentration or activity of small molecules, detect cellular processes and signaling cascades, quantify mechanical tension (a molecular "spring") (Meng et al., 2008), and monitor neuronal activity (voltage sensors), to name a few.

Recent innovations have demonstrated the use of single-molecule FRET for imaging biomolecules in live cells (Sustarsic and Kapanidis 2015), which may lead to monitoring of these processes in live animals (Hirata and Kiyokawa 2016), and molecular tension microscopy (MTM), which could monitor physical stresses or even apply forces (Gayrard and Borghi 2016) to a selected protein. Overcoming current limitations to permit the use of multiple FRET biosensors simultaneously would further increase the amount of correlated information available from a cellular process. FRET experiments are expected to continue to contribute to our understanding of basic biological processes and ultimately help develop cures for disease.

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INTRODUCTION TO FRET (CONT'D)

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TIPS FOR USING FRET IN YOUR EXPERIMENTS

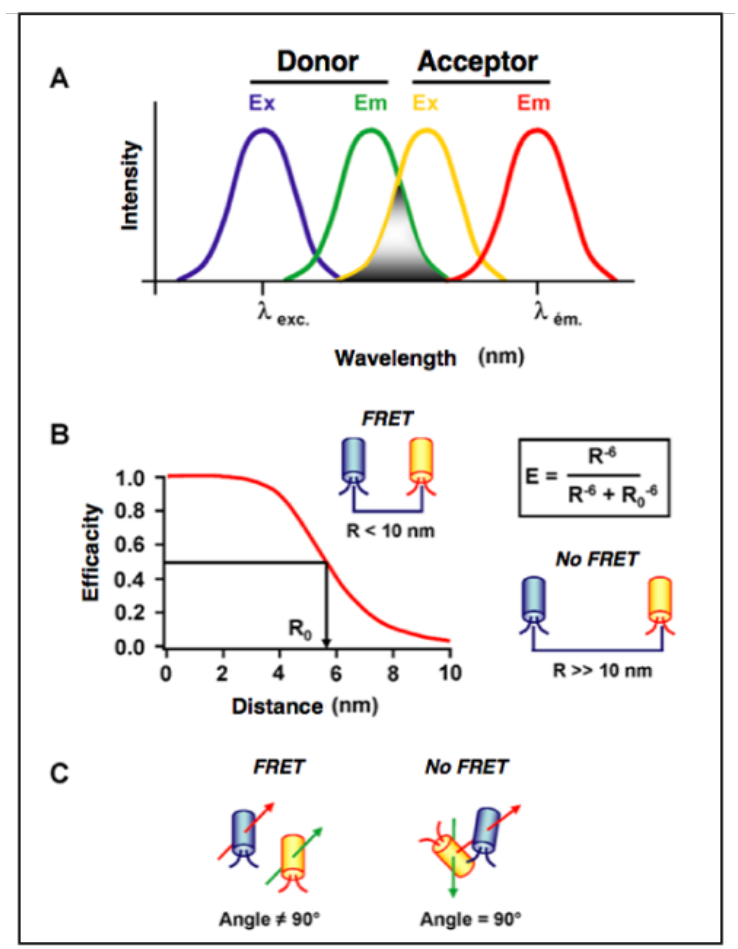
By Benoit Giquel | Nov 5, 2014

The first time I heard about FRET during a journal club, my guitarist brain automatically thought about the raised element found on the neck of my guitar... not really useful for a biologist you would say. The student was of course talking about the now well-known FRET, aka Fluorescence (Förster) Resonance Energy Transfer, technique which allows the detection of molecules' interactions, modifications or dissociations *in situ*. Used since the mid-90s, this technique has revolutionised the way we understand molecular complexes and is still a very useful tool.

Like a guitar hero (that I'm not), FRET loves playing "live". Indeed, FRET was one of the first techniques which enabled the measurement of single molecule interactions in living cells using a microscope. Historically, molecular interactions were detected by indirect means often using probes with the potential to target several molecules. By analogy, it was like pointing out a group of students in a university hall but not knowing if these students know or interact with each other. FRET reduced the scale of our perception about molecular interactions.

What is FRET?

In their JCB 2003 paper, Sekar and Periasamy defined FRET as "a distance-dependent physical process by which energy is transferred non-radiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-range dipole-dipole coupling." The emission of the acceptor fluorophore can be measured using microscopy techniques. FRET measurement sensitivity makes it suitable for studying interactions within living cells. By coupling fluorophores to proteins, pioneers of this technique were able to directly detect protein/protein interactions in living cells. Since then FRET has also been used to measure conformational changes, cleavage activity using FRET-based biosensors, and interactions between DNA and proteins. FRET, in theory, is an easy technique, but it is very important to follow some simple rules to avoid common pitfalls and to use it at its best.



Modified from Wikipedia, original author Maurel Damien. Accessed 11/4/2014.

What Are the Parameters that Affect FRET?

FRET occurs when the two fluorophores used are in close vicinity. Thus, the **distance between the two fluorophores and their orientation to one another** can affect FRET. When it comes to studying an unknown interaction between two proteins, these parameters are difficult to overcome but they have

TIPS FOR USING FRET IN YOUR EXPERIMENTS (CONT'D)

to be considered when analysing data from your FRET experiments. You may have to design several different constructs to find one that could be used for the purpose of your studies. These parameters are less important when designing a biosensor, as the distance between the two fluorophores and their spatial orientation will be fixed.

The **quantum yield of the donor** (number of emitted photons per absorbed photons) and the **extinction coefficient of the acceptor** (linking the quantity of absorbed light, at a given wavelength, to the concentration of fluorophore in solution) are two additional parameters that can affect FRET efficiency. These issues can be overcome by choosing a complementary pair of fluorophores. To maximize the FRET signal you should choose the highest quantum yield donor, the highest absorbing acceptor and fluorophores with significant overlap in their spectra. The pair CFP-YFP was the first to be used to study protein-protein interactions and several other pairs have been used since - including: mCerulean/mVenus, mCerulean/Amber, mCerulean/SYFP2A, mTurquoise/mVenus and others. CFP-YFP is still one of the best and most used pairs to measure FRET.

The table below lists plasmids that can be used to create your choice of fluorescent fusion protein with your gene of interest:

Plasmid	Color	Expression	Description
pPROEX Aqua	Cyan	Bacterial	Expresses Aquamarine with N-terminal His tag
pAquaN1	Cyan	Mammalian	Expresses mammalian optimized Aquamarine
mCerulean N1	Cyan	Mammalian	Express a gene of interest fused to the N-terminus of monomeric Cerulean
mCerulean C1	Cyan	Mammalian	Express a gene of interest fused to the C-terminus of monomeric Cerulean
mTurquoise2	Cyan	Mammalian	Constructs to target mTurquoise2 to various subcellular compartments
pCEP4Cy-Pet-MAMM	Cyan	Mammalian	Expresses mammalian optimized CyPet
pCyPet-His	Cyan	Bacterial	Expresses CyPet with C-terminal His tag
SCFP3A	Cyan	Mammalian	Express a gene of interest fused to the C-terminus of SCFP3A
Amber N1	Yellow	Mammalian	Express a gene of interest fused to the N-terminus of Amber
Amber C1	Yellow	Mammalian	Express a gene of interest fused to the C-terminus of Amber
mVenus N1	Yellow	Mammalian	Express a gene of interest fused to the N-terminus of monomeric Venus
mVenus C1	Yellow	Mammalian	Express a gene of interest fused to the C-terminus of monomeric Venus
pCEP4Y-Pet-MAMM	Yellow	Mammalian	Expresses mammalian optimized YPet
pYPet-His	Yellow	Bacterial	Expresses YPet with C-terminal His tag
SYFP2	Yellow	Mammalian	Express a gene of interest fused to the C-terminus of SYFP2

TIPS FOR USING FRET IN YOUR EXPERIMENTS (CONT'D)

Plasmid	Color	Expression	Description
Clover	Green	Mammalian	Expresses Clover (a GFP variant) commonly used with mRuby2
pLSSmOrange-N1	Orange	Mammalian	Express a gene of interest fused to the N-terminus of LSSmOrange
pLSSmOrange-C1	Orange	Mammalian	Express a gene of interest fused to the C-terminus of LSSmOrange
mRuby2	Red	Mammalian	Expresses mRuby2 (a RFP variant) commonly used with Clover
pGWF1	Cyan & Yellow	Bacterial	Gateway-compatible vector to express a gene of interest fused between ECFP and Venus

Other issues that can affect FRET measurements include: the brightness of a fluorophore pair, donor:acceptor stoichiometry, and cross-talk between the two fluorophore colours. Experts in FRET recommend to using two fluorophores that have a similar brightness - even though in the most popular pair, CFP has five-fold less brightness than YFP. The stoichiometry of donor:acceptor is difficult to address especially when you investigate unknown molecular complexes. But it is a parameter that you have to keep in mind when analysing FRET results. The cross-talk between the two fluorophores is linked to their excitation spectrum overlap. If you choose a pair too close to each other in the spectrum, you can easily directly excite the acceptor with the laser used to excite the donor. In that case the cross-talk is high and your background signal may be higher than the signal generated by the energy transfer. On the contrary, if you choose a pair that are too far from each other, you don't have any cross-talk but you also don't have any resonance transfer. You have to find a balance between FRET efficiency and cross-talk.

Methods to Measure FRET for Cell Biology Studies

Several methods have been used over the past 20 years to measure FRET and there is not one that is better than another. It is often recommended by FRET experts to use as many measurement methods as feasible when first beginning to establish the FRET methodology for a given experiment. You can then choose the most efficient approach for your own system.

The simplest and the most popular one is the sensitised emission method, where the donor is excited by a specific wavelength of light and the signal is collected by using emission filters chosen for the donor fluorescence and the acceptor fluorescence. Additionally, this method could be the best option if there is no cross-talk between FRET pairs. Unfortunately cross-talk between fluorophores does exist in the real world and corrective approaches and appropriate controls are required to make this method useful for dynamic experiments in which FRET changes are large.

Two other methods are commonly used to measure FRET: the acceptor photobleaching method and the fluorescence lifetime imaging microscopy (FLIM) method.

The acceptor photobleaching method is simple but limited to a single measurement. This method is based on the fact that the donor is quenched when FRET occurs. By photobleaching the acceptor, you release the donor's quenching and the fluorescence of the donor is increased. This method is straightforward and quantitative, but it is destructive and cannot be used for dynamic measurements. Extra care should be taken so as not to destroy the donor molecule.

TIPS FOR USING FRET IN YOUR EXPERIMENTS (CONT'D)

FLIM has been developed more recently and is the most rigorous method for measuring FRET. FLIM measures the fluorescence decay time of the donor. When FRET occurs between the pairs, donor fluorescence is quenched and the fluorescence decay time of the donor is shortened, allowing FLIM to give an unambiguous value of FRET efficiency. As you don't measure acceptor fluorescence, this method is also less sensitive to direct acceptor excitation artifacts and it is possible to use a non-fluorescent acceptor. It should be noted that FLIM is a slower imaging method, limiting its use in many FRET experiments. In addition, other environmental factors, such as pH or autofluorescence background, can change the fluorescence decay time and have to be taken into account when interpreting data.

FRET is 20 years old but as you can imagine not old-fashioned at all. With the development of new fluorescent pairs and the emergence of faster measurement systems, this technique has still many good days and good "gigs" ahead... No offense to BRET, CRET and other RET techniques which have never outclassed the Master.

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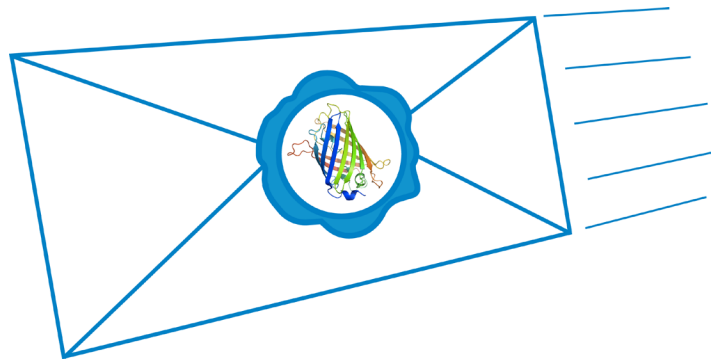
SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES

By James D. Fessenden | July 19, 2016

Biochemists often struggle to understand how a protein of interest actually behaves. How large is it? What parts of it move when you feed it substrate or add an essential cofactor? How many binding partners does it have and how do they come off and on in a cellular environment? If these are pressing issues in your laboratory, then FRET experiments are a viable biophysical path to answers.



What is FRET and What is It Good for?

Fluorescence resonance energy transfer (FRET) is a biophysical interaction between two fluorophores. When the first fluorophore (the donor) is excited, it can transfer energy directly via resonance to the second fluorophore (the acceptor) if the two probes are close together (typically 30-100 Å, depending on the probes). In fact, FRET can be used to derive a precise physical distance between the donor/acceptor fluorophores (Stryer, 1978), a measurement that is unaffected by intervening proteins, lipid bilayers, organelles or other cellular impediments.

FRET has been used in many applications, including drug discovery by big pharma (i.e. HTRF and LanthaScreen) (Degorce et al., 2009), measuring intracellular calcium (Miyawaki, Griesbeck, Heim, & Tsien, 1999), monitoring kinase activity (Ni, Titov, & Zhang, 2006), quantifying antibody/antigen interactions (Saraheimo et al., 2013), and visualizing structural changes in the ribosome during protein translation (Ermolenko et al., 2007). All that is required is that the proteins/nucleic acids/lipids of interest are fluorophore labeled and that these fluorophores can undergo FRET when they are near each other.

How do you label your biomolecule of interest with FRET fluorophores? When is absolute labeling specificity needed and when is it not? What are the proper controls needed to pressure-test your FRET assay to make sure it is working? This article addresses these questions, which are critical in setting up a FRET-based assay or screen.

Molecular Mailbox: How to Get Your Fluorophore Delivered to the Right Place?

FRET experiments require targeting the donor and acceptor fluorophores to the different biomolecules you'd like to test for interaction. In fact, targeting a FRET fluorophore to a biomolecule is a bit like a Fedex delivery. Your fluorophore package needs to go to the proper mailbox (i.e., a binding site) in the right city (protein). In a cell, this task is even harder due to tens of thousands of biomolecules each containing multiple binding crevices that can nonspecifically soak up your fluorophore package.

One important concept in FRET experimental design is that the targeting specificity requirements are different for the donor and acceptor fluorophores. The donor must be delivered to an exact spot (a single mailbox at a specific house in your molecular city). However, the acceptor doesn't need to go to the same exact location as the donor. As long as some of the acceptor molecules are targeted nearby (such as a neighboring house on the same street as the donor), then FRET measurements are possible. In fact, even if the majority of FRET

SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES (CONT'D)

acceptors are delivered to the wrong address in a different city, you can still develop a successful FRET-based assay since energy transfer only occurs when donor and acceptor are close together. The binding of acceptors >100 Å away from the donor doesn't result in energy transfer and thus, your specific FRET signal is unaffected

Due to these fundamental differences in targeting specificity for donor and acceptor fluorophores, it's best to consider these cases separately when talking about the tools that are available. Let's consider the donor first where labeling specificity is most important.

Donor Labeling and the Need for Specificity

Donor fluorophores need to go to the right place. Mis-targeted donors never "see" FRET acceptors, and thus never undergo FRET. If this nonspecific labeling far exceeds properly targeted donor/acceptor pairs, then any measureable FRET will be swamped out by a sea of uncoupled donors. The best method for targeting a fluorescent donor to your protein is to hardwire the molecule directly into the protein's peptide sequence. Thus, all synthesized target protein molecules contain the donor and, more importantly there is no fluorescence background resulting from nonspecific donor labeling.

Fluorescent proteins (FPs) are the gold standard for genetically-encoded FRET donors (Fig. 1). Scores of FP variants have been created, thereby enabling a broad spectrum of donor fluorescence wavelengths for FRET experiments. However, remember that, if you make an FP fusion, you are inserting about 20-25 kDa of protein mass into your protein and so structural changes due to this bulky insertion are inevitable. Most FP fusions for FRET studies are at the N- or C-terminus of your target protein, but even these modifications can affect the structure or function of certain proteins.

Rather than shove a bulky FP into your protein, you can site-specifically label it with finesse using tRNA suppression technology (Fig. 1) (Dumas, Lercher, Spicer, & Davis, 2015). By using a novel aminoacyl tRNA synthetase/tRNA pair, a researcher can lure the ribosome into inserting a fluorophore-tagged amino acid at a stop codon (often amber i.e. UAG) engineered into a desired site. During translation, the novel tRNA/unnatural amino acid finds the UAG codon in the ribosome, resulting in site-specific incorporation of the attached fluorophore. However, since tRNA suppression competes with natural chain termination at stop codons, expression levels of these tagged proteins are often very low. In addition, a major overhaul of the cell's native tRNA synthetases is required and thus far, this method has proven most effective only in a few cell types (*E. coli* and *Xenopus oocytes*, mainly). tRNA suppression plasmids are available from depositing labs that have mastered this technique so it might be worth a try, especially since the potential rewards could be substantial:

Plasmid	tRNA Synthetase	Expression Host
pMAH-POLY	Polyspecific aminoacyl-tRNA Synthetase	Mammalian Cells
pDULE-ABK	pyrrolysyl-tRNA synthase for aliphatic diazirine amino acids	*E. coli* and Mammalian Cells
pEVOL-pAzF	synthetase for p-azido-l-phenylalanine	*E. coli*
pEVOL-pBpF	synthetase for p-benzoyl-l-phenylalanine	*E. coli*
pAcBac1.tR4-MbPyl	pyrrolysyl-tRNA synthetase	Mammalian Cells
pCMV-DnpK	dinitrophenyl hapten	Mammalian Cells
pANAP	AnapRS	Mammalian Cells

SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES (CONT'D)

Acceptor Labeling: Sometimes It's Okay to Be Wrong

In contrast to FRET donors, absolute FRET acceptor labeling specificity is not always necessary for successful FRET experiments. If the acceptor fluorophore is getting to a molecular mailbox that is close to the donor, faulty delivery to other locations in the cell are not a problem, as long as they are beyond the FRET range of the donor (i.e. >100 Å). Researchers have developed an extensive array of chemical labeling strategies suitable for targeting FRET acceptors to proteins (reviewed in Yan & Bruchez, 2015). To get you started, two robust orthogonal labeling strategies are presented (Fig. 1) which can be carried out using Addgene plasmids.

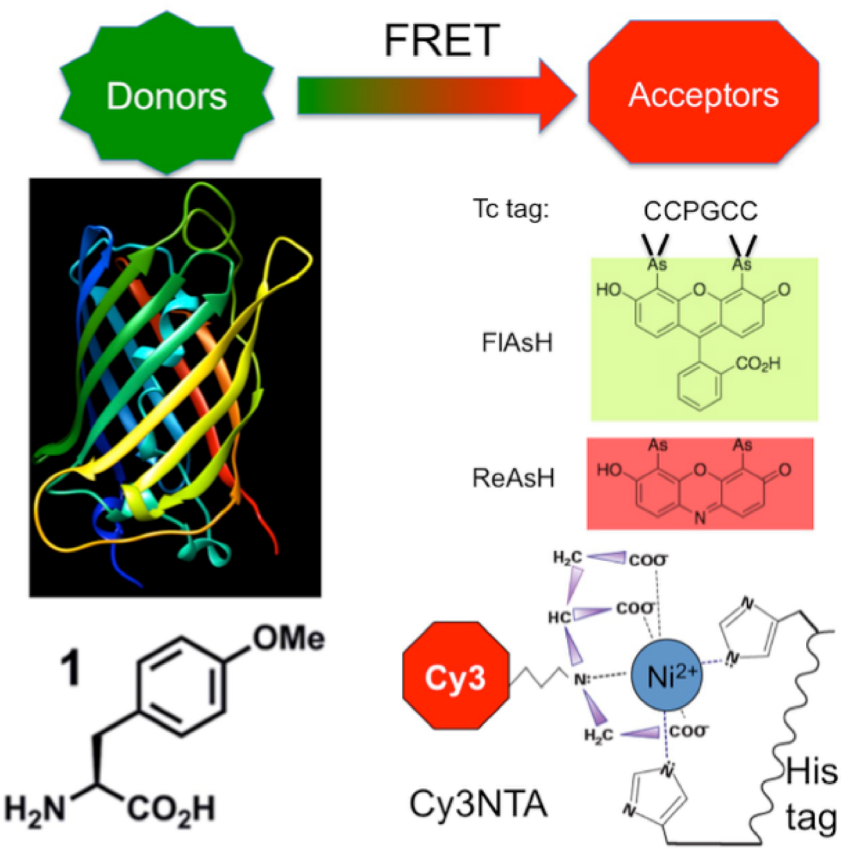


Figure 1: FRET donors such as fluorescent proteins (left, top) and fluorescent unnatural amino acids (left, bottom) can be genetically encoded into a target protein for maximum labeling specificity. Robust FRET acceptors such as biarsenicals (right, top) and Cy/NTA dyes (right, bottom) can be delivered to short target sequences inserted into a target protein. Unnatural amino acid image modified from (Dumas et al., 2014).

His-tag Labeling Reagents

You may already have a FRET acceptor-binding site on your protein of interest: you just don't know it yet! The poly-his tag often used for protein purification is a reliable FRET acceptor labeling site when used in conjunction with Cy dye-nitrilotriacetic acid (NTA) conjugates (Kapanidis, Ebright, & Ebright, 2001). These Cy/NTA conjugates work the same way as NTA-sepharose beads used for protein purification: the NTA binds to the His tag on your protein and the coupled Cy fluorophore acts as the FRET acceptor. Binding affinity can be enhanced by coupling two NTA molecules to each Cy dye, as well as by extending the length of the his tag from 6 to 10 residues. While these Cy/NTA conjugates are not commercially available, they are easily synthesized with reagents used to fluorescently label antibodies, and purification is a snap using thin layer chromatography (Fessenden, 2009). However, these reagents can bind endogenous biomolecules in cells nonspecifically and they cannot cross cell membranes, so they are best used either with plasma membrane proteins or with experiments on permeabilized cells.

Biarsenical Labeling Reagents

If you want to label intracellular proteins covalently with FRET acceptors, then biarsenicals are a good option. Originally developed by Roger Tsien and co-workers (Griffin, Adams, & Tsien, 1998), these dyes are now commercially available as Lumio Green and Lumio Red from Invitrogen (now ThermoFisher Scientific). These reagents are nonfluorescent until they bind a 6-residue tetracysteine tag (sequence: CCPGCC) that can be inserted into the target protein. The utility of these reagents is that binding is covalent, so free dye can be

SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES (CONT'D)

washed away. In addition, labeling contrast can be improved using disulfide-containing compounds such as British anti-Lewisite (which was originally developed to treat heavy metal intoxication in chemical warfare [Vilensky & Redman, 2003]). Most importantly, these compounds cross cell membranes, thus enabling FRET measurements in intact cells. The Gradia Lab has deposited a gateway vector with Addgene, where you can insert your protein of interest, resulting in a C-terminal fusion of a tetracysteine tag. In addition, since these tags are so small, one can insert them in virtually any desired location in a target protein, though predicted unstructured loops are best since these tags form beta-hairpins (Madani et al., 2009).

Donor and Acceptors: Putting it All Together

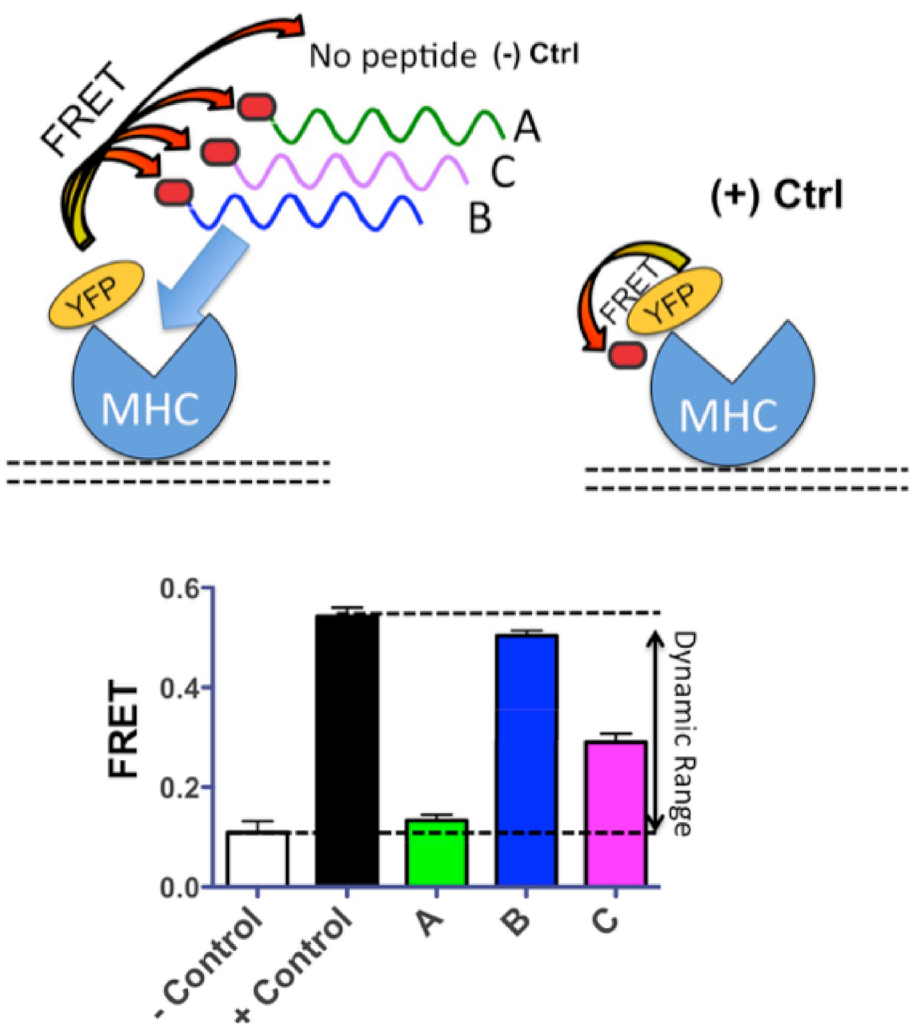


Figure 2: Hypothetical FRET-based assay to monitor binding of a library of His-tagged peptides to the major histocompatibility complex (MHC) used for T-cell development. FRET between a YFP-tagged MHC expressed on the cell surface and Cy3NTA bound to His-tagged antigenic peptides (upper left) or no peptide (negative control) is monitored. A positive control construct consisting of a Cy3NTA bound, His-tagged YFP fused to the MHC provides the upper limit for FRET measurements in this assay. The dynamic range of the assay is bounded by FRET to the negative and positive control experiments (lower panel). Of three sample peptides, A does not bind to MHC, B binds closest to the YFP fusion and C is at an intermediate location. Follow-up experiments using this assay could include determination of binding kinetics, affinity measurements and site-directed mutagenesis to identify amino acid residues required for these protein-peptide interactions.

The FRET donors and acceptors described above can be paired together to measure a wide range of molecular distances (see Table). However, proper control experiments are needed to establish the dynamic range of your assay. These experiments include producing a positive control construct where the donor and the acceptor fluorophore binding sites are adjacent to each other on the same protein and a negative control experiment conducted on donor-only labeled protein that is incubated with your FRET acceptor. These experiments will define the maximum and minimum energy transfer values you can expect, thereby establishing the dynamic range of your FRET assay (Figure 2).

These labeling methods are freely interchangeable and new uses for FRET are continually being developed, so don't be afraid to try novel, unpublished combinations of labeling strategies. FRET experimental design lends itself to creativity and innovation, valuable commodities in any NIH grant application! Using FRET, you may gain a new structural understanding of your protein that can lead to novel insights about its biology and its behavior. See the table below for donor and acceptor pairs that you can use in your FRET experiments.

SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES (CONT'D)

Donor	Acceptor	Tag	Ro (Å)[a]	Range (Å)[b]	Applications
CFP	Lumio Green	Tc: CCPGCC	50	40-72	Intact Cells
	Cy3NTA[c]	Poly His	44	35-63	Plasma membrane protein, permeabilized cells
	Cy5NTA[c]		32	25-45	
GFP (fluores-cein is similar)	Lumio Red	Tc: CCPGCC	48	38-70	Intact Cells
	Cy3NTA[c]	Poly His	63	50-90	Plasma membrane protein, permeabilized cells
	Cy5NTA[c]		43	34-62	
YFP	Lumio Red	Tc: CCPGCC	54	42-78	Intact Cells
	Cy3NTA[c]	Poly His	65	52-94	Plasma membrane protein, permeabilized cells
	Cy5NTA[c]		59	47-85	

[a] R0 is the donor/acceptor (D/A) distance at which 50% FRET occurs.

[b] Range corresponds to calculated D/A distances for observed FRET efficiencies between 80% and 10%.

[C] Cy3NTA and Cy5NTA bind His tags similarly but with differing R0 values, thereby enabling calibrated FRET distance measurements.

Further Reading

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SPECIAL DELIVERY: FLUOROPHORE TARGETING FOR FRET STUDIES (CONT'D)

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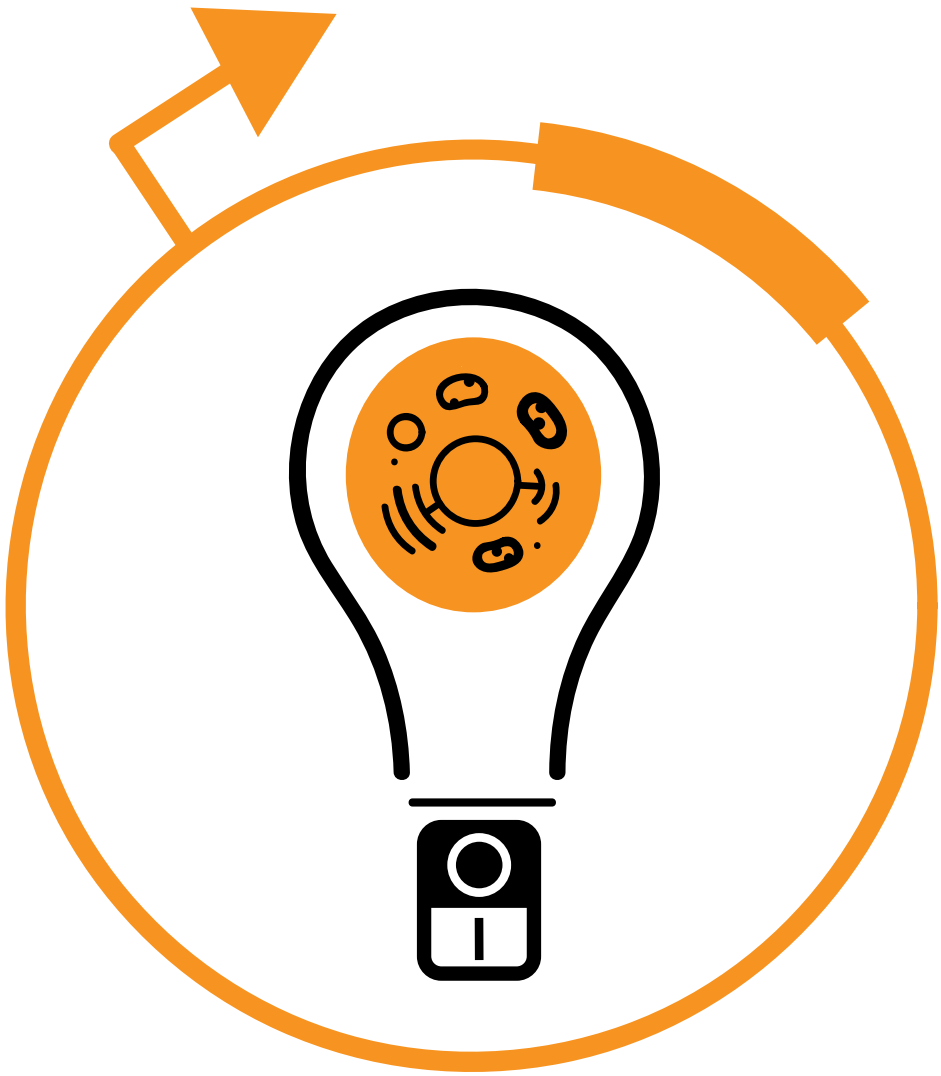
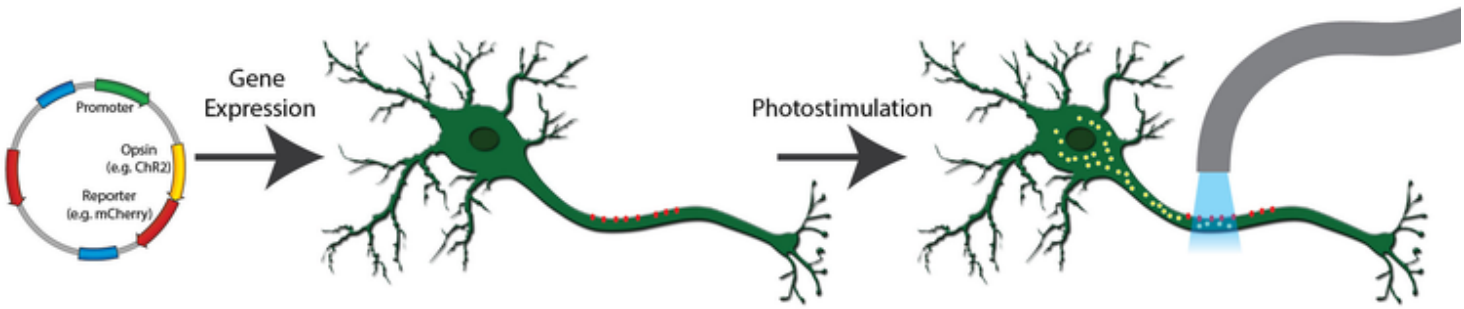
CHAPTER 5: OPTOGENETICS



INTRODUCTION TO OPTOGENETICS

By Caroline LaManna | July 19, 2017

The field of optogenetics integrates optics and genetic engineering to measure and manipulate cells (frequently neurons) and their governing biomolecular processes. The tools and technologies developed for optogenetics research utilize light to detect, measure, and control molecular signals and cells in order to understand their functions.



Example schematic of an optogenetics procedure. A channelrhodopsin, fused to mCherry, is expressed in neurons (red dots). When exposed to light of the correct wavelength, the pore opens, cations flow into the cell (yellow dots), and the neuron is activated.

Optogenetics tools can be broadly classified based on their functions into two groups:

- **Actuators** are genetically-encoded tools for light-activated control of proteins; e.g., microbial opsins and optical switches
- **Sensors** are genetically-encoded reporters of molecular signals; e.g., calcium indicators

In this overview, we will focus on the common actuators used in optogenetics. For information on sensors, check out our biosensors collection.

Microbial Opsins

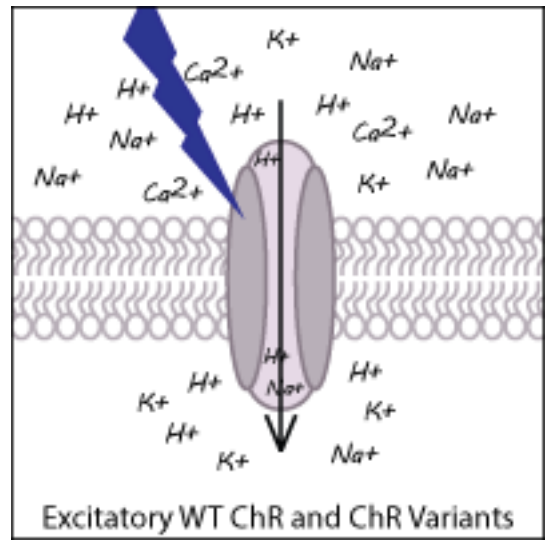
Opsins are light-gated ion channels or pumps that absorb light at specific wavelengths. Upon activation by light, these channels and pumps respond by opening or closing, which conducts the flow of ions into or out of the cell. Scientists have identified a variety of naturally occurring microbial opsins that respond to different wavelengths of light, like blue or yellow light. These various opsins also initiate different electrochemical responses, such as nonspecific cation influx vs. proton efflux. Researchers have used genetic engineering to improve these natural opsins - by inducing point mutations to alter the absorption spectrum or adding trafficking signals to localize opsins to the cell membrane.

Microbial opsins, such as those described below, can be targeted and expressed in specific subsets of neurons, allowing precise spatiotemporal control of these neurons by turning on and off the light source. Optogenetics has been broadly applied to study the physiology of the brain and nervous system to better map and understand neuronal circuits. Optogenetic tools have also been used to trigger and study certain behavioral responses in model organisms like mice, zebrafish, and *Drosophila*. These tools have been instrumental in neurological disorder research, helping scientists to better understand Parkinson's disease, depression, drug addiction, and more.

Below is a list of commonly used microbial opsins, including a brief overview of the wild-type opsin of each and some examples of relevant variants. This list is not exhaustive - please browse our curated list of opsins here to find the right optogenetics plasmids for your experiments.

INTRODUCTION TO OPTOGENETICS (CONT'D)

Channelrhodopsins



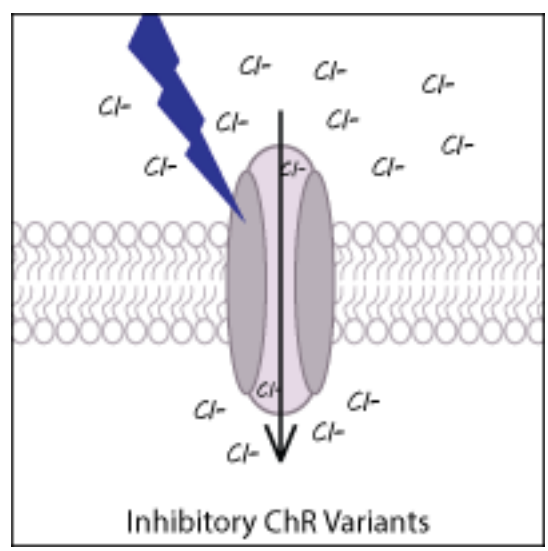
Channelrhodopsins are foundational optogenetics tools - they typically allow the fast depolarization of neurons upon exposure to light through direct stimulation of ion channels. Naturally occurring channelrhodopsins were discovered in the green algae *Chlamydomonas reinhardtii*. Channelrhodopsin-1 (ChR1) is excited by blue light and permits nonspecific cation influx into the cell when stimulated. Channelrhodopsin-2 (ChR2), the first widely adopted optogenetic tool, is also a blue light activated cation channel. ChR2 is preferred over ChR1 because ChR2 has higher conductance at physiological pH and trafficks well to the membrane.

- **Excitatory (Depolarizing) ChR Variants** - The optogenetic toolbox has been expanded by scientists through both the identification of novel ChRs from other algal species and the development of synthetic variants to enhance the functionality of ChR. Examples of ChRs from other species include: CsChR (from *Chloromonas subdivisa*), CoChR (from *Chloromonas oogama*), and SdChR (from *Scherffelia dubia*). Synthetic variants have been created via genetic point mutations, codon optimization, and chimeric fusion of domains from two different ChRs. These ChR variants still function as light-gated, cation channels resulting in excitation (depolarization) of the neuron. Feature enhancements include:

  -Increased photocurrent amplitude
  Examples: ChR2(H134R), C1V1(t/t), ChIEF

  -Increased channel (on/off) kinetics
  Examples: ChETA, C1V1(t/t), ChrimsonR

  -Red-shifted peak action spectra
  Examples: VChR1, C1V1(t/t), Chrimson, ChrimsonR, Chronos



- **Inhibitory (Hyperpolarizing) ChR Variants** - Alternatively, ChR variants that inhibit neurons have been created and identified in other species - by acting as light-gated chloride channels, these variants result in the hyperpolarization of neurons. Examples of anion channel variants from other species include: GtACR1 and GtACR2 (from the cryptophyte *Guillardia theta*). Other feature enhancements include:

  -Increased photocurrent amplitude
  Examples: iChloC, SwiChRca

Browse Channelrhodospin plasmids.

Halorhodopsins

Halorhodopsins are light-gated inward chloride pumps isolated from halobacteria. Wild-type halorhodopsin,

INTRODUCTION TO OPTOGENETICS (CONT'D)

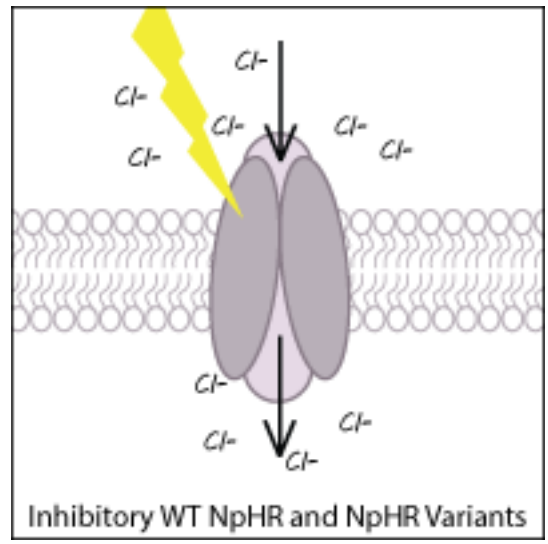
known as NpHR (from *Natronomonas pharaoni*), causes hyperpolarization (inhibition) of the cell when triggered with yellow light, thus inhibiting function of the neuron.

- **NpHR Variants** - Variants have been engineered with enhancements such as:

  -Human codon optimization
  Example: Halo

  -Increased photocurrent amplitude
  Examples: eNpHR, eNpHR2.0, eNpHR3.0

  -Red-shifted peak action spectra
  Example: Jaws



Browse Halorhodopsin plasmids.

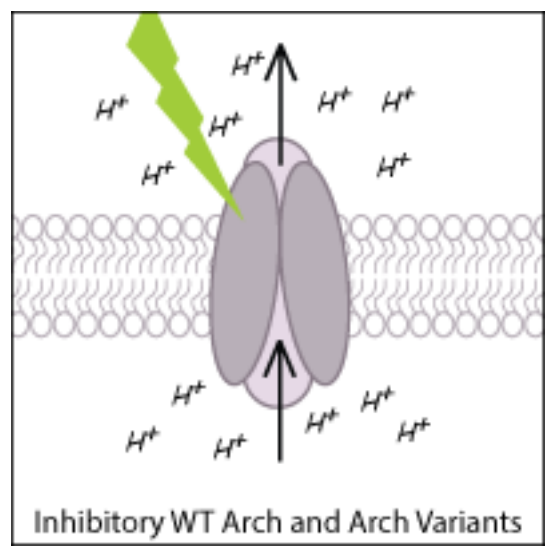
Archaerhodopsins

Archaerhodopsin-3 (Arch) from *Halorubrum sodomense* is also commonly used to inhibit neurons in optogenetic experiments. Arch is a light-activated outward proton pump that hyperpolarizes (inhibits) the cell when triggered by green-yellow light.

- **Arch Variants** - Arch variants have been developed with the following enhancements:

  -Increased light sensitivity
  Example: ArchT

  -Increased photocurrent amplitude
  Examples: eArch3.0, eArchT3.0



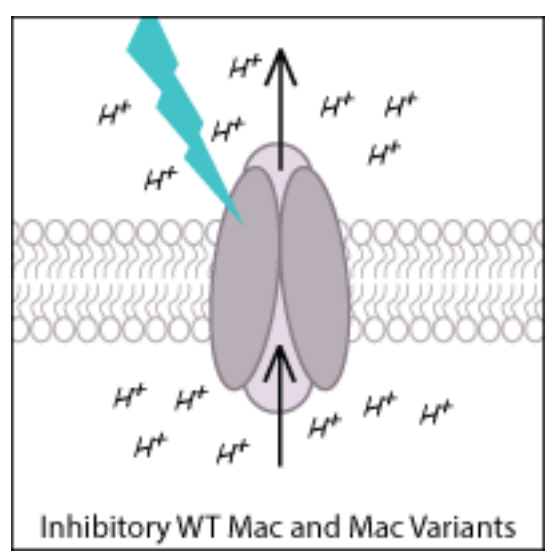
Browse Archaerhodopsin plasmids.

Wild-Type Mac

Leptosphaeria rhodopsin (Mac) is a blue-green light-activated proton pump derived from the fungus *Leptosphaeria maculans*. Mac and its variants allow for inhibition of neurons using blue-green light.

- **Mac Variants** - Mac variants have been engineered to include enhancements such as:

  -Improved photocurrent amplitude
  Example: eMac3.0



Browse Leptosphaeria plasmids.

INTRODUCTION TO OPTOGENETICS (CONT'D)

Plan Your Optogenetics Experiment

When designing your optogenetics experiment, you'll need to pick both an opsin and a delivery system. Here are some key factors to consider:

Optogenetic excitation or optogenetic inhibition. First things first: do you want to turn ON or turn OFF neurons in your experiment? Depending on your answer, you'd pick an excitatory or inhibitory opsin, respectively.

Color of activation light. There are a variety of different activation wavelengths, ranging from blue to yellow to red. Red light exhibits better tissue penetrance, which may allow you to place the optic fiber outside of the brain, rendering the experimental procedure less invasive. Different activation wavelengths also make it possible to combine multiple opsins in the same experiment. For example, you could use different colors of light to activate/silence the same neuronal population, or activate different neural populations at distinct times.

Temporal considerations. Temporal precision is key in optogenetic experiments. Your experimental design will determine whether you'll need short or long periods of neuronal activation/inactivation periods. These can range from msec (hChR2) to "long lasting," e.g. seconds to minutes with stable step-function opsins (SSFOs).

Opsin delivery systems. Two factors determine which neuronal population is manipulated in a given experiment: the expression of the opsin and the area that is being illuminated. There are several different ways to control opsin expression. Generally, the most robust and stable expression is achieved using a transgenic mouse line, such as the VGAT-ChR2 mouse, where ChR2 is expressed under the control of the vesicular gamma aminobutyric acid (GABA) transporter (VGAT) and ChR2 is thus expressed in all GABAergic neurons. In this case, the subpopulation of GABAergic neurons being activated by the light is controlled via the placement of the optic fiber.

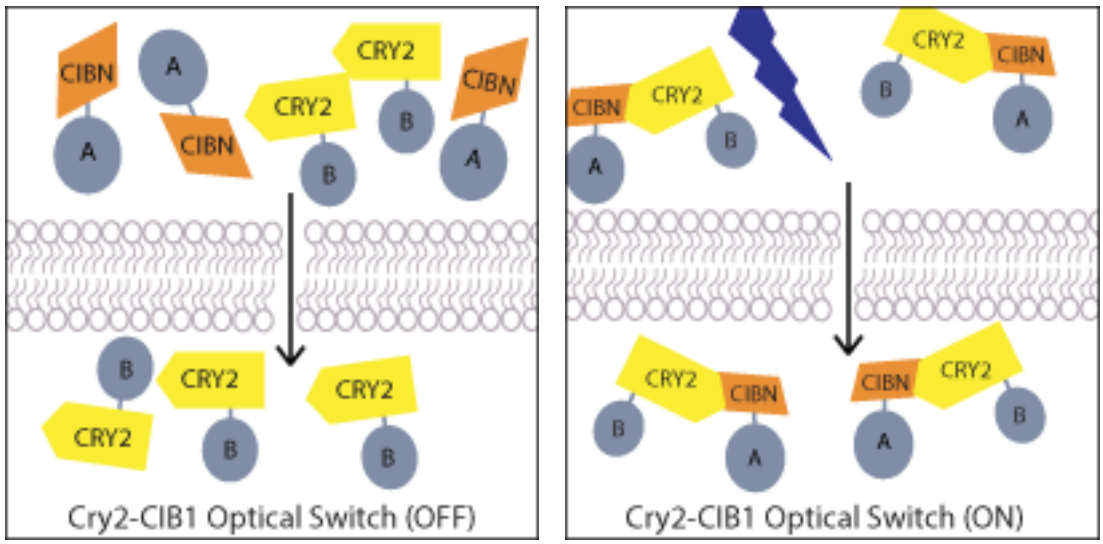
Using viral vectors for opsin delivery results in more localized expression of the opsin. Depending on the virus and promoter system used, there is an incubation time (days to weeks) until peak expression of the opsin is reached. This approach is especially powerful when combined with site specific recombinase technology like Cre-lox, which allows you to express an opsin ONLY in a genetically defined subpopulation of cells within the injection site of the viral vector, rather than in all cells. For example, using a viral vector with a floxed opsin in a VGAT-cre animal will result in expression of the opsin only in inhibitory neurons near the injection site.

Optical Switches

Researchers have harnessed plant and bacterial photoreceptors to create protein systems controlled by light. These "photoswitchable" proteins offer exquisite spatial and temporal control of protein activity. Phytochrome, cryptochome, and LOV (light oxygen voltage)-based systems have been used in many experimental contexts, including protein activation, membrane localization, and transcriptional activation.

In the widely used Cry2-CIB1 system, light induces a conformational change in cryptochrome Cry2 to permit CIB1 binding. As shown in the figure below, this light induced binding can be utilized to control the localization of a protein of interest. A genetically-encoded nuclear Cry2 fusion can thus direct a CIB1-protein fusion to the nucleus upon light activiation. Alternatively, when one half of a given protein, such as the Cre recombinase, is fused to Cry2 and the other half to CIB1, light-stimulated heterodimerization can reconstitute the protein. This principle has also permitted the design of synthetic two-part transcription factors - Cry2 and CIB1 are fused to

INTRODUCTION TO OPTOGENETICS (CONT'D)



a transcriptional activation domain and DNA binding domain, allowing light to activate transcription. Phytochromes and light oxygen voltage (LOV) domains function similarly to cryptochromes with light-induced changes in protein conformation/dimerization. In addition to the applications described above, both LOV and Dronpa can control activity of a fused protein through allosteric interactions. Researchers continue to engineer new optobiology tools and improve upon previous tools through directed mutagenesis - use Addgene's Optical Switch Plasmid Table to search for plasmids for your next experiment. For a full description of different domains used in these plasmids, see our gloassary of optical switches.

Further Reading

1. OpenOptogenetics: the optogenetics wiki.
2. Optogenetic Switches from OptoBase, W. Weber Lab
3. Edward Boyden's Lab: Synthetic Neurobiology Group Optogenetics Resources
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SYNTHETIC PHOTOBIOLOGY: OPTOGENETICS FOR E. COLI

By Mary Gearing | Sep 8, 2015

As optogenetics turns 10 years old, it's easy to forget that this technique isn't limited to neuroscience. In fact, precise light-based control of biological processes is highly useful in other fields, including synthetic biology. Addgene depositors Christopher Voigt and Jeffrey Tabor have been working on making *E. coli* light responsive since 2005, when Tabor was working in Voigt's lab. Years later, these classic systems continue to be optimized by Tabor's lab, making light-controlled gene expression in *E. coli* easier and more robust.

Synthetic Photobiology: Why Use Light?

In synthetic biology and bioengineering, scientists seek to directly probe and build new systems. Such goals require methods to directly activate and inactivate biological processes. Chemical effectors are suboptimal for a number of reasons, including their potential toxicity and cross-reactivity with other pathways. Since these effectors are diffusible, they're also not suitable for spatially limited studies and can fluctuate with changing culture conditions.

The use of low intensity light solves each of these problems; inexpensive tools such as LEDs can be engineered to deliver precise, consistent, and controllable pulses of light. Complicated patterns of light intensity and wavelength can be used to study dynamic processes. Conversely, varied input patterns may also be used to engineer new pathways with sophisticated gene expression controls. Most cell types do not respond to low-intensity light, so off-target effects should be limited.

Phytochromes are light-responsive systems found in some bacteria, but not *E. coli*. They fall under the heading of two-component systems (TCSs). TCSs consist of a histidine kinase that phosphorylates a response regulator (RR). TCS-induced responses are varied; one outcome is directed transcription from a given promoter.

To harness the power of light, Christopher Voigt's lab created the first *E. coli* light-sensitive two-component system (TCS). Levskaya et al. fused a photosensory domain from cyanobacteria to a common *E. coli* histidine kinase. This hybrid construct senses light, and an obligate chromophore allows the system to respond to various light inputs. In far-red light or dark conditions, the chromophore activates the RR (OmpR) via phosphorylation, promoting transcription (see figure below). Subsequent exposure to red light rapidly deactivates the system. Voigt's lab used this system to develop a bacterial camera that prints a chemical image, as well as a genetic method for the computational problem of edge detection. Tabor et al. subsequently engineered a second photosensitive TCS activated by green light; these two systems can be coexpressed for sophisticated control of gene expression.

Version 1 of this far-red/red light-responsive system is spread across three plasmids, with the response regulator encoded in the genome. To make the system less bulky and easier to use with other strains of *E. coli*, Schmidl et al. condensed the system to two streamlined plasmids.

The utility of these TCSs was limited by a few factors. These bulky systems were spread over a number of plasmids, with one RR (ompR) encoded chromosomally. Leakiness was also an issue; some promoter activity persisted in the inactive state. The dynamic range, defined as the difference between the lowest and highest level of "output" signal, was only about 10-fold, precluding the study of gene expression at very high or very low levels.

SYNTHETIC PHOTOBIOLOGY: OPTOGENETICS FOR E. COLI (CONT'D)

Schmidl et al. chose to optimize these systems to make them more tunable and user-friendly. They experimented with the promoter strength of various components, removing inducible promoters that could crosstalk with other pathways. They also reduced system leakiness and increased dynamic range to around 100-fold. These improvements will allow researchers to better fine-tune the level of output gene expression based on the input light intensity. In addition to these improvements, the constructs have been greatly streamlined; only two plasmids are required per system.

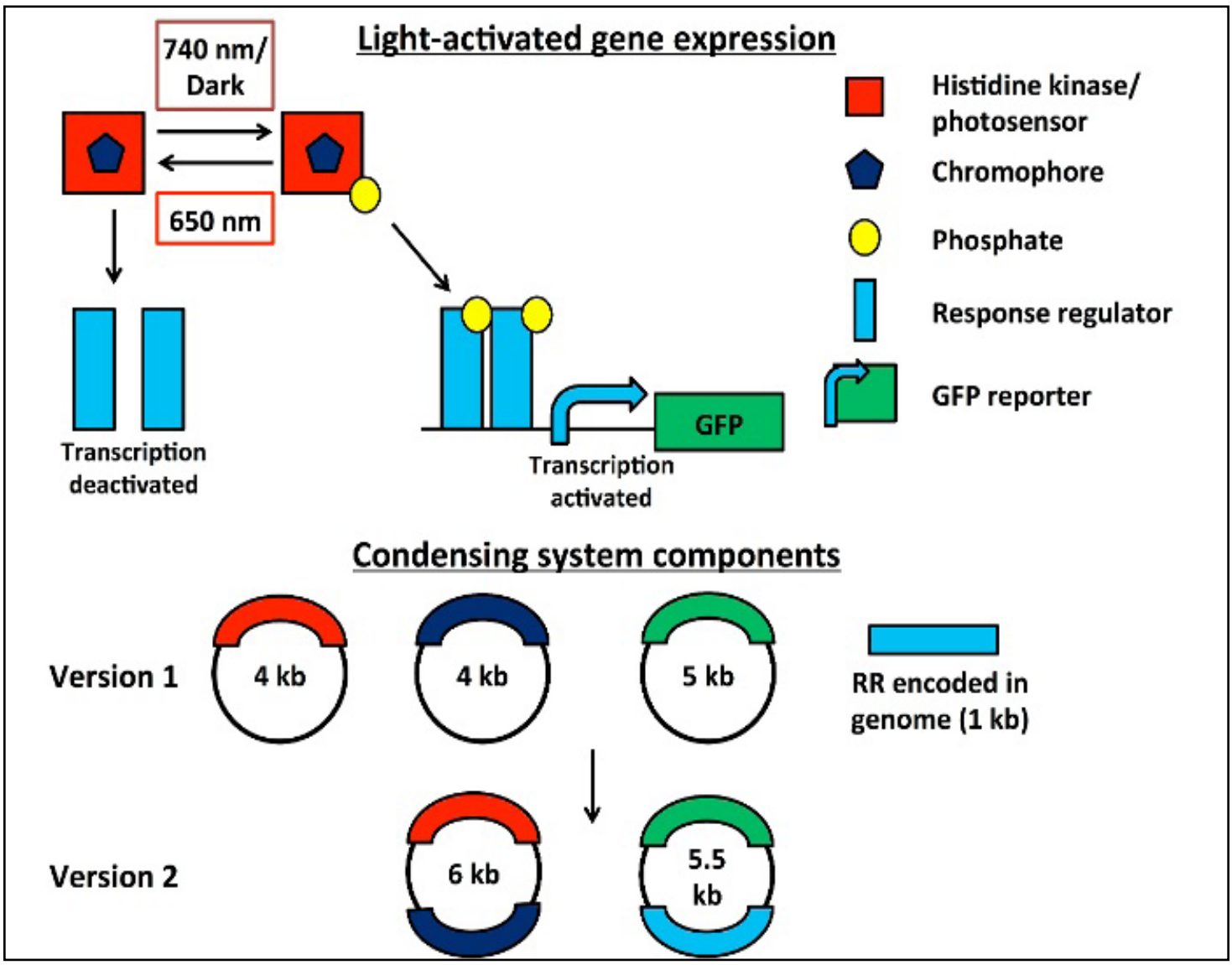

Figure 1: An E. coli light-sensitive two-component system (TCS). A hybrid histidine kinase/photosensor senses various light inputs, and an obligate chromophore allows the system to respond. In far-red light or dark conditions, the chromophore activates the response regulator via phosphorylation, promoting transcription of a GFP reporter. Subsequent exposure to red light rapidly deactivates the system.

SYNTHETIC PHOTOBIOLOGY: OPTOGENETICS FOR E. COLI (CONT'D)

Applications of these Systems

The large dynamic range of these systems ensures that they can be used with many types of proteins, including Cas9 and dCas9-transcription factors, for which low expression levels are optimal. These tools are compatible with many strains of *E. coli*, as well as with other plasmid-based systems, which will enable the assembly of larger systems.

The work of Schmidl et al. also represents a large step forward in optimizing TCSs. Over 75,000 TCSs have been identified in bacteria, and with similar optimization methods, many of these systems could be made suitable for various scientific or engineering applications.

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OPTOGENETICS + CRISPR: USING LIGHT TO CONTROL GENOME EDITING

By Caroline LaManna | August 26, 2015

Scientists around the world have been making major improvements to CRISPR technology since its initial applications for genome engineering in 2012. (Check out our CRISPR 101 eBook for everything you need to know about CRISPR.) Like CRISPR, optogenetics has also been making headlines over the past decade. Optogenetics uses genetically encoded tools, such as microbial opsins, to control cellular activities using light. In 2015, scientists combined CRISPR and optogenetics techniques to develop a variety of photoactivatable CRISPR tools. These tools allow scientists to use light to externally control the location, timing, and reversibility of the genome editing process. Read on to learn about the various light-controlled CRISPR tools available to researchers - some readily found at Addgene.

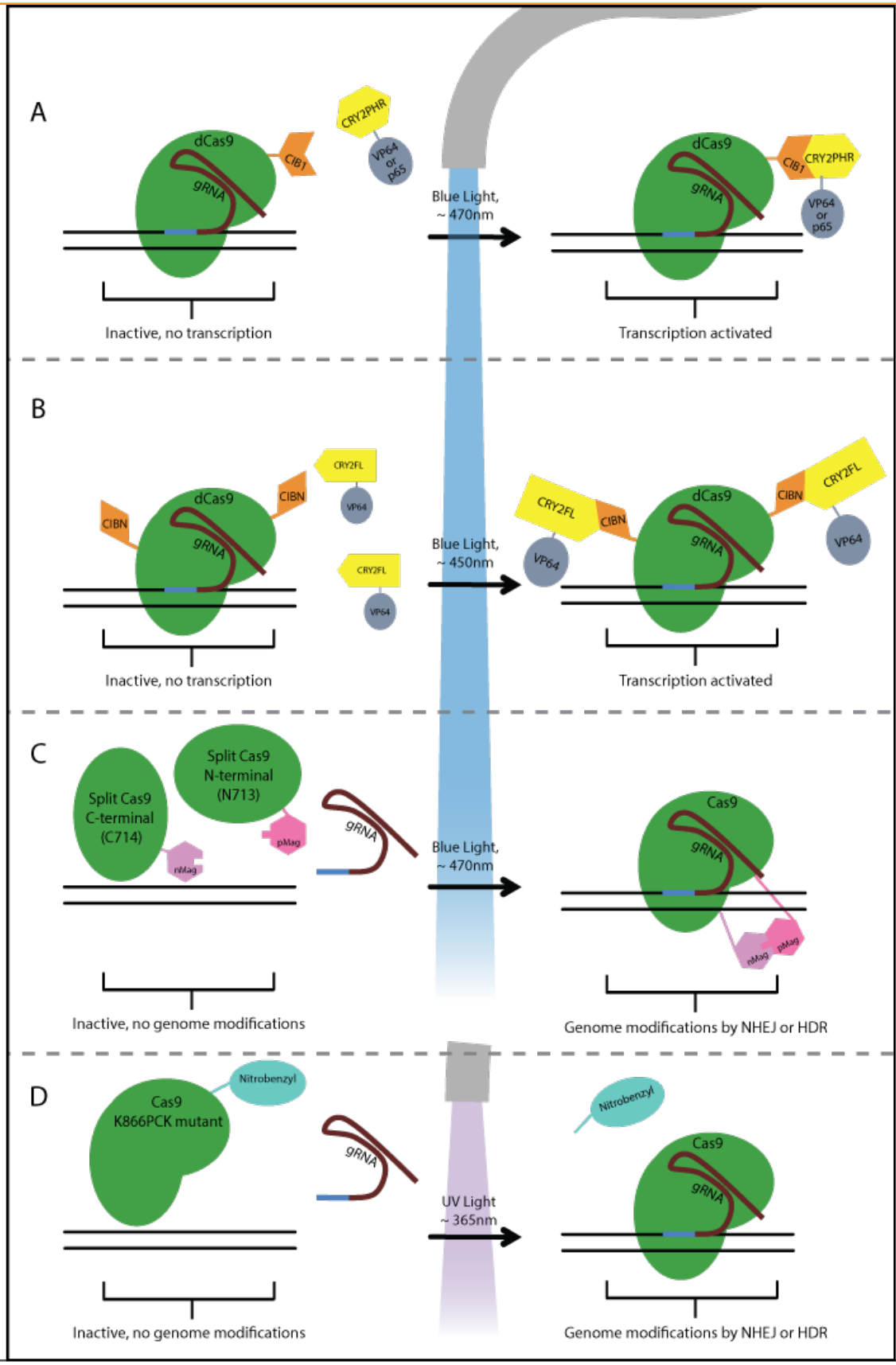
Shining Light on Transcriptional Activation Using dCas9

Initial photoactivatable CRISPR systems published in early 2015 focused on using light to control transcription. Two separate labs, Moritoshi Sato's lab at the University of Tokyo (Nihongaki Y, et al., Chemistry & Biology, 2015 Feb 19; 22(2):169-74) and Charles Gersbach's lab at Duke University (Polstein LR, et al., Nature Chemical Biology, 2015 Mar; 11(3): 198-200) developed similar systems based on the light-inducible heterodimerizing cryptochrome 2 (CRY2) and calcium and integrin-binding protein 1 (CIB1) proteins. The goal of both groups was to create a system that would use light to turn on and off gene expression while imparting spatiotemporal control, reversibility, and repeatability.

The system developed by Nihongaki et al. is composed of two fusion proteins: 1) the genomic anchor - an inactive, dead Cas9 protein (dCas9) fused to CIB1; and 2) the activator - the CRY2 photolyase homology region (CRY2PHR) fused to a transcriptional activator domain (VP64 or p65). Upon expression in the cell, the dCas9-CIB1 fusion binds to the target DNA sequence as directed by the guide RNA (gRNA), while the CRY2PHR-activator fusion floats freely, depicted in the figure below (A). Once triggered by blue light, the CRY2 and CIB1 proteins heterodimerize and move the activator into position to activate gene transcription. The researchers tested a variety of combinations to optimize both fusion proteins, including making alterations to the CIB1 domain, testing various activator probes, and adding various genomic anchors to the N-terminus of both fusion constructs. The best performing combination was NLS-dCas9-trCIB1 and NLSx3-CRYPHR-p65 - it had the lowest background activity in the dark state and highest fold induction at 31X. By using a slit pattern during blue light exposure (470 nm), the researchers showed that expression of the human ASCL1 gene could be spatially controlled. The authors also cycled blue light on and off and showed that ASCL1 expression followed suit - control was indeed reversible and repeatable.

With their light-activated CRISPR/Cas9 effector (LACE) system Polstein et al., utilized a similar strategy to develop an optimized photoactivatable CRISPR gene activation system, but settled on a different optimal fusion protein combination. Shown in the figure (B, next page), the optimized LACE system consisted of: 1) CIBN-dCas9-CIBN, where CIBN is the N-terminal fragment of CIB1 and it was fused to both the N- and C-termini of dCas9; and 2) CRY2FL-VP64, a fusion of full-length CRY2 and the transcriptional activator domain VP64. Using this system in HEK293T cells to induce expression of human IL1RN, the researchers saw an 11-fold increase in mRNA levels after 2 hr and a 400-fold increase after 30 hr. The system was also shown to be reversible and repeatable when blue light (450nm) was cycled on-off-on. Using a slit photomask, the researchers also demonstrated the ability to spatially control gene expression.

OPTOGENETICS + CRISPR: USING LIGHT TO CONTROL GENOME EDITING (CONT'D)



OPTOGENETICS + CRISPR: USING LIGHT TO CONTROL GENOME EDITING (CONT'D)

Photoactivatable Genome Modifications by NHEJ and HDR

Later in 2015, the Sato lab unveiled a photoactivatable system to cleave a target DNA sequence (Nihongaki Y, et al., Nature Biotechnology, 2015 Jul; 33(7):755-60) resulting in a double strand break (DSB) that can be repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR). This system is unique in that it utilizes a "split" nuclease - the authors fragmented Cas9 into N-terminal (residues 2-713, N713) and C-terminal (residues 714-1368, C714) halves, rendering the Cas9 non-functional when split but regaining functionality when the halves are reassociated. By fusing a photoinducible, heterodimerizing domain to each of the Cas9 fragments, the authors created a photoactive Cas9 tool, as shown in the figure (C, previous page). Although the authors tried a few different photoactivatable designs (some similar to those used in the previous Nihongaki et al. system) their most successful design utilized Magnet photoswitchable proteins derived from the fungal photoreceptor, Vivid (VVD, N. crassa) (Kawano F, et al., Nature Communications, 2015 Feb 24; 6:6256). Nicknamed paCas9-1 and consisting of the fusion proteins N713-pMag and nMagHigh1-C714, this new system had both low background and high fold-induction of Cas9 activity (16.4-fold). This paCas9-1 light-inducible system was able to recognize the same PAM and had similar targeting specificity as full-length Cas9 (flCas9). When triggered by blue light (470 nm), paCas9-1 induced indel mutations via NHEJ (frequency of 20.5%) and induced modifications by HDR (frequency of 7.2%).

The authors additionally showed that they could lower the background activity of the system by modifying paCas9-1 using nMagC714 instead of nMagHigh1-C714, generating paCas9-2. This change did not significantly alter the system's efficiency at generating mutations when activated with light and lowered background DSBs (non detectable). Like their prior work, the Sato lab also showed that the paCas9-2 system could be spatially controlled and reversibly activated by turning blue light on and off.

As one might expect from the modular nature of Cas9, Nihongaki et al. showed that it was possible to swap out the Cas9 domains in their split fusions and generate a photoactivatable nickase and a photoactivatable repressor (dCas9). The activity of all variants was reversible and repeatable.

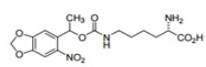
Using Chemistry to Photocage CRISPRs

The aforementioned techniques each employed a photoactive strategy which had been engineered from naturally occurring photoactive proteins (i.e. CRY2 and Vivid) - Alexander Deiters' lab, on the other hand, took a different approach. These researchers used a genetically encoded photocaging technique to insert a light-removable protecting group, specifically a nitrobenzyl photocaged lysine (PCK), on the Cas9 protein (Hemphill J, et al., JACS, 2015 May 6; 137(17):5642-5). In order to insert the PCK into a specific site on the Cas9, the group used an engineered pyrrolysyl tRNA/tRNA synthetase pair which would add the PCK upon reaching the amber stop codon, TAG (learn more about site-specific incorporation of amino acids using pyrrolysl tRNA synthetase).

The group first tested photocaging various lysines in Cas9 to determine which best deactivated the protein's ability to cleave targeted DNA, settling on photocaging the K866 lysine, as seen in the figure above (D). Next, by using a dual reporter fluorescence assay, Hemphill et al. demonstrated that the Cas9 K866PCK mutant was indeed inactive prior to irradiation with UV light (365 nm) and that post-UV exposure it showed cleavage activity similar to the wild-type Cas9. This photocaging technique was also shown to impart spatial control of Cas9 cleavage when using a photomasking technique. Last, Hemphill et al. presented data showing that this genetically encoded, photocaged Cas9 system could silence endogenous gene expression - demonstrating light-induced silencing of transferrin receptor CD71 in HeLa cells.

OPTOGENETICS + CRISPR: USING LIGHT TO CONTROL GENOME EDITING (CONT'D)

Comparison of Photoactivatable CRISPR Strategies

	Publication	System Nickname	Photoactivatable Moiety	Cas9 Variant	Genome Editing Uses
A	Nihongaki Y, et al., Chem Biol, 2015 Feb 19; 22(2):169-74		CRY2 and CIB1 proteins, from A. thaliana	dCas9	Activation of gene transcription
B	Polstein LR, et al., Nat Chem Biol, 2015 Mar; 11(3): 198-200	LACE	CRY2 and CIB1 proteins, from A. thaliana	dCas9	Activation of gene transcription
C	Nihongaki Y, et al., Nat Biotech, 2015 Jul; 33(7):755-60	paCas9	Magnet proteins, from the fungal photoreceptor Vivid (N. crassa)	• Split Cas9 • Split Cas9 nickase • Split dCas9	Genome modifications by NHEJ and HDR, repression of gene expression by CRISPRi
D	Hemphill J, et al., JACS, 2015 May; 137(17):5642-5		Nitrobenzyl caged lysine 	Cas9 K866PCK mutant	Genome modifications by NHEJ and HDR

Whether you are looking to activate, repress, or modify a gene, you now have the tools at your disposal to control your genome editing using light. We look forward to more tools as CRISPR and optogenetics continue to evolve and can't wait to see what cool applications you use these for in the future!

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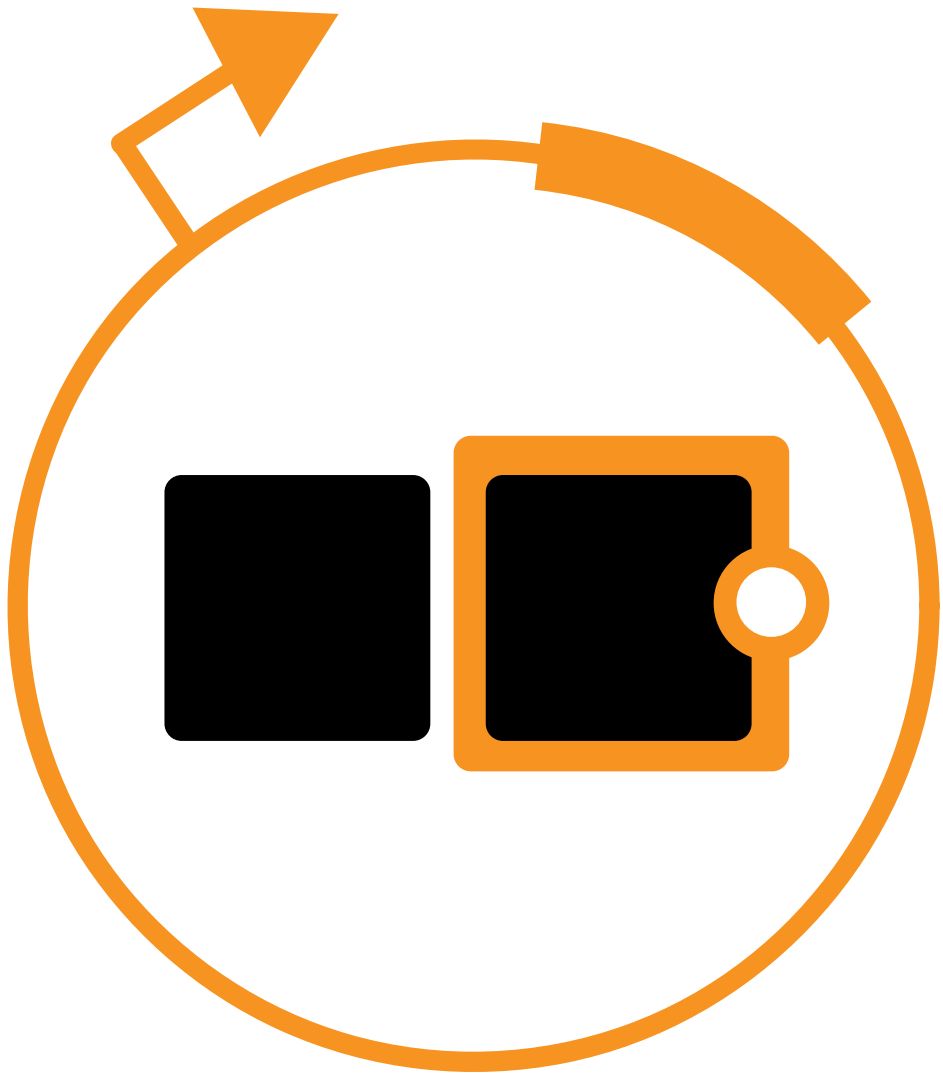
CHAPTER 6: BIOSENSORS



INTRODUCTION TO BIOSENSORS

By Jessica Welch | May 15, 2017

Biosensors ('biological sensors') are biological tools that monitor a process or detect a given molecule. The sensor component is usually a protein which undergoes a conformational change in response to the molecule it detects. This change then generates a reporter signal. Reporter signals may be electrochemical or light-based, with luminescent and fluorescent reporters being especially popular. We'll give you an introduction to fluorescent biosensors, but keep in mind that there is a lot of variety in how biosensors work, and you should always check the associated publication for the specifics of your chosen plasmid.

Types of Biosensors

When choosing a biosensor, you first need to decide the best way to track or measure the process you're interested in. Depending on the process you would like to monitor, there may be multiple steps you could examine, i.e. for an enzymatic reaction, you could measure consumption of a substrate OR production of a product OR enzyme activity.

Small Molecule Biosensors

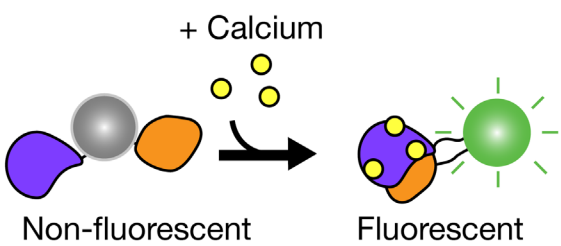


Figure 1: Generic example of a plasmid-encoded fluorescent biosensor for calcium. The protein fusion is non-fluorescent until a calcium binding module (purple) binds to calcium ions (yellow) and binds a second domain in the fusion protein (orange). This binding rearranges the fluorescent protein module of the fusion protein (grey/green) such that it is able to fluoresce.

Addgene has many sensors that measure the concentration of small molecules like arabinose or hydrogen peroxide or ions like calcium and zinc. Some biosensors contain a single fluorescent protein fused to a domain that senses the concentration of a given ion - we'll use calcium biosensors as an example. Many calcium biosensors include GFP fused to calmodulin (CaM) and the chicken myosin kinase calmodulin binding region M13. When calcium is absent, the construct does not fluoresce; when present, calcium induces CaM/M13 interaction, thereby changing the FP's structural organization and activating the fluorescent signal. Blue flame GECO biosensors from Robert Campbell's lab display calcium-dependent increases in fluorescence of up to 11,000-fold.

FRET biosensors are another common class of biosensors. Blue flame "cameleon" calcium sensors created by Roger Tsien's lab contain two fluorescent proteins (a FRET pair) separated by CaM and M13. When calcium binds, it triggers CaM/M13 interaction that increases FRET between the fluorescent proteins. FRET is highly amenable to biosensor creation, and custom FRET biosensors can be created using the cpFRET kit created by Olivier Pertz's lab.

Other biosensors track cellular characteristics like pH, redox state, and voltage across membranes. These sensors generally follow the same pattern described above - a sensing domain induces changes in a fluorescent signal based on ligand presence or changing cellular conditions.

Other Types of Biosensors

To focus on a particular gene or protein, check out our gene-specific biosensors. These constructs are often used to monitor enzymatic activity or phosphorylation over time. You can also use biosensors to follow

INTRODUCTION TO BIOSENSORS (CONT'D)

processes such as protein or DNA binding, enzyme or transcriptional activation, conformational changes, translation, and protein translocation.

Targeting Your Biosensor

Before expressing a biosensor in eukaryotic cells, think about the best place for the biosensor to be expressed. Are you looking at an intracellular or extracellular molecule? By fusing a biosensor to an organelle-specific targeting signal or a transmembrane domain, biosensors can be targeted to a particular cellular compartment such as the nucleus, mitochondria, endoplasmic reticulum (ER), vacuole, or cellular membranes. Examples include blue flame mitochondria-targeted calcium sensor CMV-mito-GEM-GECO1 and blue flame extracellular glutamate sensor pCMV(MinDis).iGluSnFR.

Picking Your Reporter

Don't forget about the other end of the biosensor: which fluorescent protein is most compatible with your lab and experimental setup? Consider what kind of instrumentation you have available, and the conditions of your experiment. For example, does the fluorescent tag need to be pH stable or withstand a particular solute concentration or temperature? For further advice, see our posts on how to select a fluorescent protein for single and multi-color imaging experiments.

Finally, what kind of system will you use to detect your fluorescent protein? The type of detection method depends on what type of information you are looking for. Below we have summarized some of the common fluorescent detection methods and the types of experiments they are commonly used for.

Experiment	Detection Method	Notes
Quantification of a Process	Plate-based assays (plate reader)	Detect the average amount of fluorescence across a population of cells (i.e. in a plate well). Use to track a process (e.g. enzyme activity) over time or under various conditions
Localization within Cells	Fluorescence microscopy	Visualize components of individual cells
Separation/ identification of fluorescently-labeled components	Fluorescence-activated cell sorting (for cells); fluorescent HPLC (proteins or other molecules)	Isolate or quantify cells, proteins or other molecules that have been labelled with your fluorescent molecule

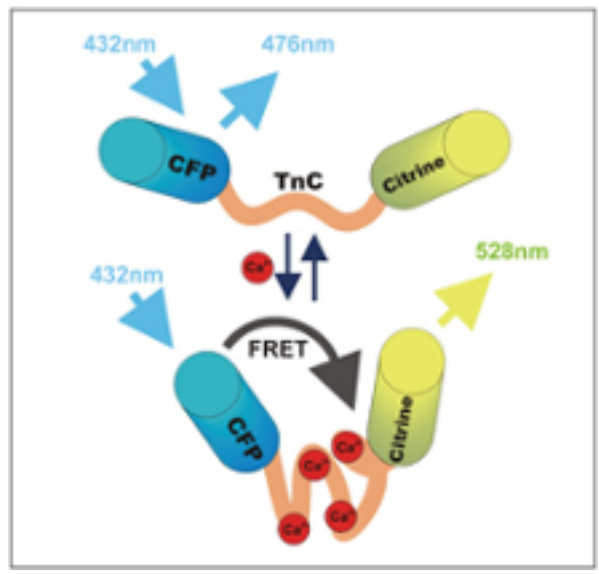
FRET BASED BIOSENSORS

By Kendall Morgan | May 6, 2014

Oliver Griesbeck of the Max Planck Institute for Neurobiology has been working on genetically encoded indicators of calcium and other small molecules since the very beginnings of the field. Those engineered sensors were designed to replace synthetic calcium dyes, which had been in use since the 1980s.

"Synthetic dyes were the standard in the field, but there is one problem: how to get that into the cells of interest," Griesbeck said. Because they are chemical compounds, they have to be applied or injected, and they don't always end up where you want them to go.

Griesbeck is motivated by a particular interest in monitoring the activity and biochemistry of living neurons in an effort to understand the connection between molecular- and cellular-level events and behavior. It's a problem that he considers "one of the greatest challenges of neuroscience."



FRET based biosensor from the Griesbeck lab.

Twitch Sensors

Griesbeck's recent contribution to the field is a series of fluorescence resonance energy transfer (FRET)-based calcium biosensors. Perhaps most notably, the Twitch calcium sensors, which are available at Addgene, can be used for ratiometric *in vivo* imaging. Griesbeck explains, while these Twitch sensors are always bright, they change color as they shift from "off" to "on" in the presence of calcium. This color change can be observed under a microscope using commercially available beam splitters. In addition to imaging neurons, Griesbeck adds that this color change has a particular advantage for observing events taking place inside cells that are on the move, such as T lymphocytes.

Griesbeck and his colleagues began by isolating a calcium-binding protein, troponin C (TnC), from muscle cells. They identified a toadfish TnC variant which had a high-affinity for calcium binding and developed a version of the protein with as few calcium-binding sites as possible for added sensitivity.

As reported in Nature Methods, the FRET changes of these Twitch sensor variants were optimized by testing various linker configurations in a large-scale functional screen in bacteria. Sensor variants were then refined by a secondary screen in rat hippocampal neuron cultures. The optimized Twitch sensors allowed the researchers to see tonic action potential firing in neurons and high resolution functional tracking of T lymphocytes, too, making them a versatile tool for application in both neuroscience and immunology.

Ratiometric Imaging for Beginners

Griesbeck says his calcium indicators can be used in mouse, *Drosophila*, *C. elegans*, and zebrafish – essentially all of the standard organisms for which extensive genetic tools are available. While he recognizes that some beginners may have a tendency to shy away from ratiometric imaging, he recommends they give it a shot.

"Beginners sometimes think it's complicated, but actually it is very good," he said. "It gives you more information than intensity-based readouts."

FRET BASED BIOSENSORS (CONT'D)

Griesbeck also points out that the approach he and his colleagues took to optimize sensitivities can now be applied to dozens of other published FRET-based sensors. For example, his team is now applying it to improve upon an indicator they developed and published in 2010 for visualizing the transcription factor cAMP-responsive element-binding protein (CREB) in living cells.

"Now we are making this really good," Griesbeck said. "We know CREB is really important, but where is it really important and when? That is not known."

Transition Metals

Maarten Merkx of Technische Universiteit Eindhoven has similar interest in FRET-based sensor proteins, particularly for the intracellular imaging of transition metal ions, such as zinc, copper, and iron.

"These are essential metal ions, but they are also toxic," Merkx explained. New tools were needed to measure them.

Merkx says, while in principle adapting a FRET sensor for monitoring calcium versus zinc should require the simple swapping of binding domains, in practice optimizing the sensors involves plenty of trial and error. Transition metals also present challenges in that they are found in cells at much lower concentrations than calcium, requiring greater sensitivity.

He and his colleagues came up with a solution: they devised self-associating fluorescent domains whose association is disrupted in the presence of a ligand (they stick together in one state, but not the other).

"It's quite a robust way to make a FRET sensor with a large dynamic range," he says. Merkx recently applied this "trick" to the development of additional color variants. This resulted in the construction of redCALWY-1, a red-shifted FRET sensor for zinc using variants of mOrange2 and mCherry as donor and acceptor domains. He says, these new colors might now enable observation of the interaction between different molecules like zinc and calcium.

Ultimately, Merkx said he hopes others will find his tools useful, including his zinc sensor and his genetically encoded magnesium sensor, the first of its kind.

"I hope by depositing at Addgene, people will start using them," Merkx said. "For us, it is one of the main measures of success – whether we have really developed something useful [enough] that other people start using it. For us, it's like a test."

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ILLUMINATING EPIGENETICS WITH A FRET BASED BIOSENSOR

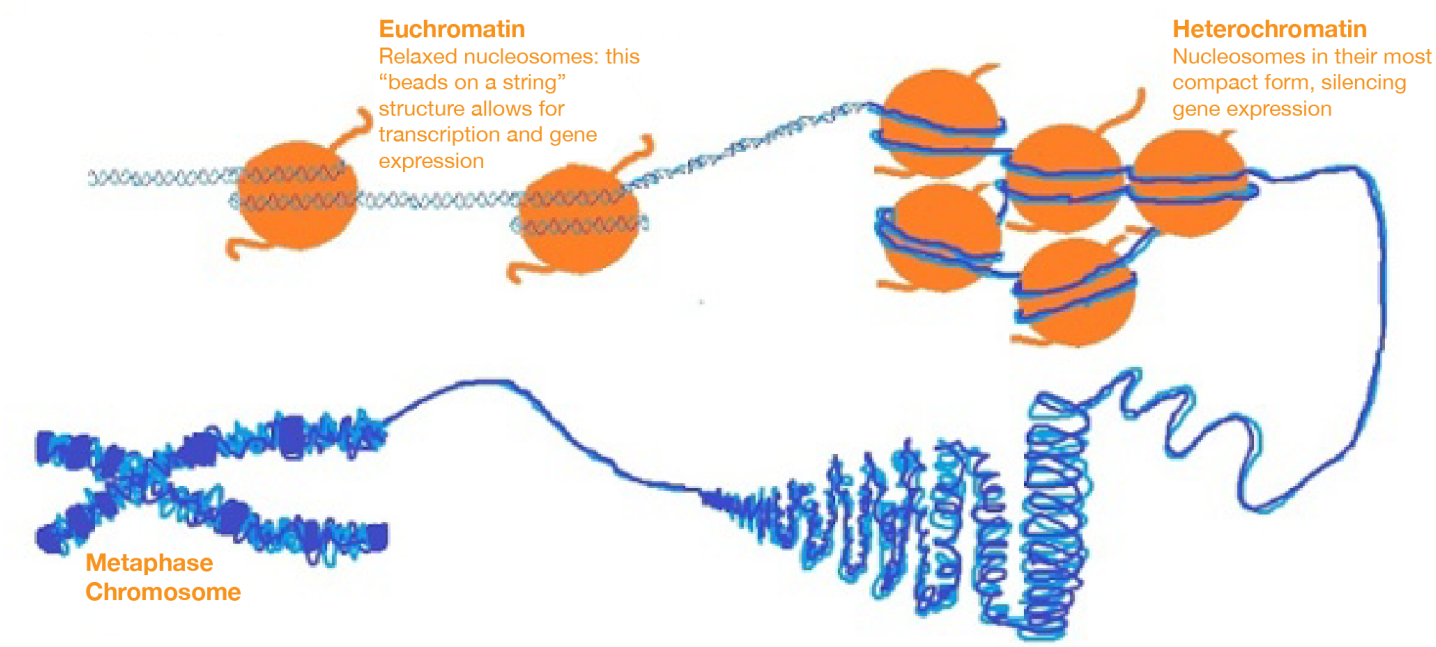
By Emma Markham | November 19, 2015

Epigenetics has recently been hitting the headlines, with stories like the potential devastation of the palm oil industry through epigenetic effects on the Cover of Nature. So what is epigenetics and what tools are available to study it?

What Exactly is Epigenetics?

Epigenetics is the study of heritable changes in gene expression that do not involve any changes to DNA sequence (Epigenetics literally means 'on top of the genome'). Epigenetic modifications can result in phenotypic changes without any change in genotype. Epigenetic modifications play a large role in turning genes 'off' and 'on', signaling which genes should be 'read' by the cellular machinery. This is how most cells in the body contain the same DNA but have very different structure and function, and can have a specialized role within an organism.

Overview of the Compression of Chromatin within the Chromosome
When viewed through a light microscope, the chromosome is made up of dark and light bands. The dark bands correspond to highly compressed areas of histones and DNA, whereas the light bands are composed of more open structures. The leve of gene expression is directly related to the level of compaction.



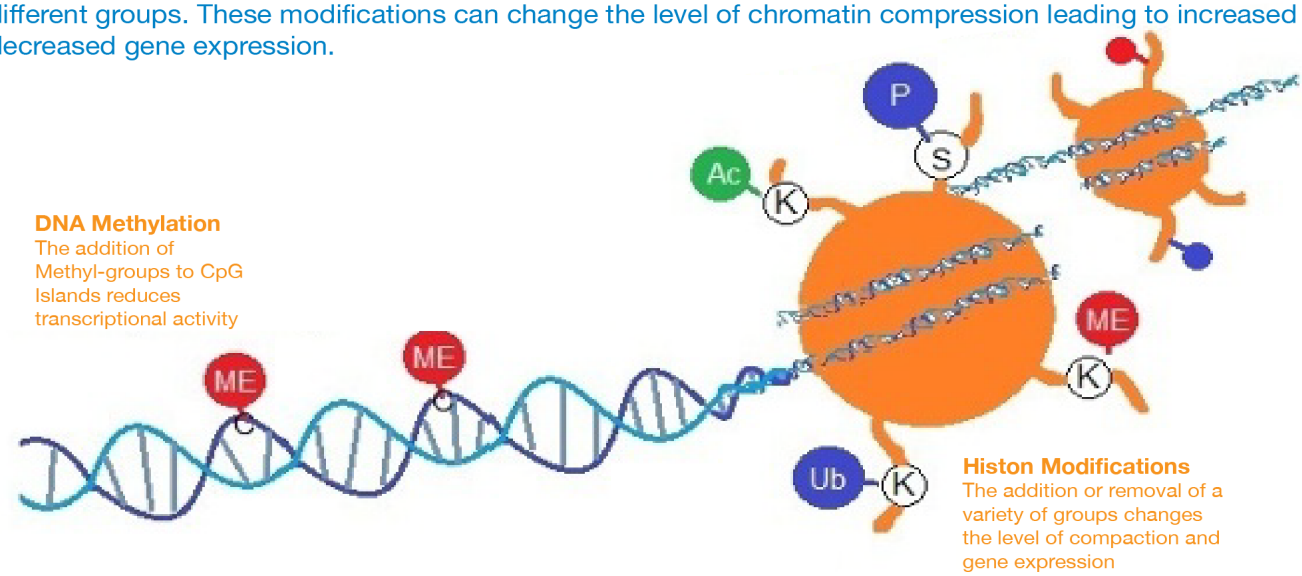
Mechanisms of Epigenetic Modifications

The main mechanism of epigenetic modification is chromatin remodeling. Chromatin is a complex of DNA and histone proteins that compacts cellular DNA into chromosomes and reduces the risk of dangerous DNA damage. If the way DNA is wrapped around histones changes, then gene expression can change as well. The more frequently and tightly bound DNA is to histones, the more compacted it becomes. This prevents the cellular machinery from physically interacting with the genes in the compacted region and results in a loss of gene expression. In this way, genes in compacted regions are essentially 'silenced'.

ILLUMINATING EPIGENETICS WITH A FRET BASED BIOSENSOR (CONT'D)

Modifications that Influence Gene Expression

DNA methylation physically blocks physically blocks the transcription machinery from interacting with the DNA thereby stopping gene expression. Histones can be modified in a range of ways by the addition or removal of different groups. These modifications can change the level of chromatin compression leading to increased or decreased gene expression.



Chromatin remodeling can occur by two main mechanisms, either through histone modifications, which can influence the transcription of genes by RNA polymerase II, or by the addition of methyl- groups to the DNA, which mostly occurs at CpG islands (regions of the DNA with a high frequency of C and G nucleotides). The conversion of cytosine to 5-methylcytosine in CpG islands makes genes less transcriptionally active.

Histone Modifcations and Their Functions (Non-exhaustive)

Histone Site		Modifcation	Function	Histone Site		Modification	Function
H1	S27	Phosphorylation	Activation	H3	K9	Acetylation	Activation
	K26	Methylation	Silencing		K14	Acetylation	Activation
H2A	K5	Acetylation	Activation		K18	Acetylation	Activation
	K7	Acetylation	Activation		K23	Acetylation	Activation
H2B	K5	Acetylation	Activation		K27	Acetylation	Activation
	K11	Acetylation	Activation		K4	Methylation	Activation
	K12	Acetylation	Activation		R8	Methylation	Silencing
	K15	Acetylation	Activation		K9	Methylation	Silencing
	K16	Acetylation	Activation		R17	Methylation	Activation
	K20	Acetylation	Activation		K27	Methylation	Silencing
H4	K5	Acetylation	Activation		K36	Methylation	Activation
	K8	Acetylation	Activation		K79	Methylation	Activation
	K12	Acetylation	Activation		S10	Phosphorylation	Activation
	K16	Acetylation	Activation				
	R3	Methylation	Activation				
	K59	Methylation	Silencing				

ILLUMINATING EPIGENETICS WITH A FRET BASED BIOSENSOR (CONT'D)

The Role of Epigenetics in Studying Disease

Epigenetic changes have been discovered to play a large role in a variety of diseases, ranging from cancer, to diabetes, to congenital disorders.

A series of studies carried out on an isolated population in Sweden found that paternal grandfathers who had experienced famine before their adolescence passed on a heritable trait to their grandsons that decreased their likelihood of death from cardiovascular disease but increased their risk of death from diabetes. Conversely, paternal grandmothers who experienced famine while still in their mother's womb passed on a trait to their granddaughters that reduced their lifespan. Because these dramatic conditions were experienced in a period of life when the grandparents' gametes were developing, an epigenetic change is thought to have occurred that was passed down to the grandchildren; in this study, epigenetic changes were statistically significantly associated with reduced life-span, but no changes in the DNA sequence could be found.

Angelman syndrome and Prader–Willi syndrome, which have very different disease phenotypes, are two fascinating examples of epigenetics in the form of imprinting. In imprinting, one copy of a gene (either the maternal or paternal copy) is silenced by the addition of methyl groups, and so only the paternal or maternal copy of the gene is expressed in normal individuals (in a parent-of-origin-specific manner), but the expression or silencing of both copies causes disease because too much or too little gene product disrupts normal function.

Angelman syndrome is characterized by intellectual and developmental disability, jerky movements, and spontaneous laughter or smiling, whereas Prader–Willi syndrome is characterized by low muscle tone, short stature, cognitive disabilities, problem behaviors, and a chronic feeling of hunger that can lead to excessive eating and obesity. What is fascinating is that both syndromes can present without any DNA changes, and both involve the same region of chromosome 15. They both result from the inactivation of genes on one copy of chromosome 15 (the maternal copy for Angelman syndrome and the paternal for Prader-Willi syndrome). In both cases, the other copy of the chromosome is normally imprinted and silenced, and so the syndromes are caused by a lack of the corresponding gene products due to the same region, but not the same exact genes, being improperly silenced in both cases. In both diseases, genes from both copies of chromosome 15 are inactivated, but different phenotypes result depending upon whether the improperly imprinted genes come from the mother or father.

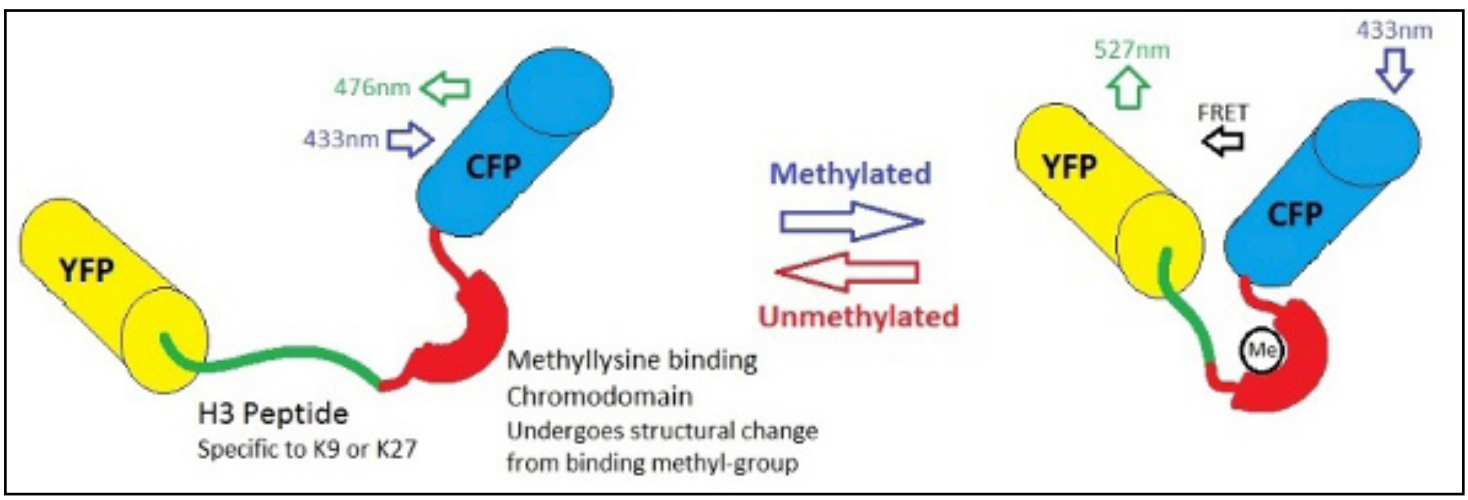
Many cancers have dramatic changes in their DNA methylation patterns, either large increases or decreases. Hypermethylation of CpG islands in the promoter region of tumor suppressor genes can repress their transcription and inactivate them. Global hypomethylation, on the other hand, has also been implicated in the development and progression of cancer through different mechanisms.

New FRET Based Tools to Study Epigenetics

Epigenetic research uses a wide range of molecular biologic techniques to further the understanding of epigenetics; techniques include chromatin immunoprecipitation, fluorescent in-situ hybridization, methylation-sensitive restriction enzyme digestion, DNA adenine methyltransferase identification, and bisulfite sequencing. However none of these techniques can be used for *in-situ* research of dynamic modifications in real-time, posing a huge challenge to studying epigenetic changes over time. This challenge was difficult to overcome until now.

ILLUMINATING EPIGENETICS WITH A FRET BASED BIOSENSOR (CONT'D)

Alice Ting's lab has developed a new, plasmid-based biosensor to visualise changes in histone methylation in living cells. Essentially this reporter construct consists of one substrate (the histone-derived peptide) which can localise the construct specifically to the histone protein of interest (in the figure above, H3 at K9 or K27). Joined to this by a flexible linker is a methyllysine binding domain (chromodomain), which binds selectively to lysine-methylated peptides. This construct is in the middle of a FRET pair, which is used to visualise the results. When methylation occurs at the histone-derived peptide, the methyllysine binding domain causes a structural change (facilitated by the flexible linker) that brings the fluorescent units of the FRET pair together and causes an increase in the FRET signal. Subsequent demethylation separates the fluorescent units and so lowers the FRET signal back again.



Ting Lab construct consisting of histone-specific binding domain and the FRET reporter.

The Ting lab constructed 2 versions of this reporter to target the K9 (pcDNA3-K9 histone methylation reporter) and K27 (pcDNA3-K27 histone methylation reporter) positions on histone H3, which are known to be involved in repression and X-inactivation. The K9 reporter uses the HP1 chromodomain to recognise lysine 9 methylation, while the K27 reporter uses the polycomb chromodomain to recognise lysine 27 methylation. When tested in living cells where methylation of these sites was controlled, the reporter consistently showed significantly different FRET levels between unmethylated and methylated states. Thus, this system is a reliable tool in reporting methylation status of these sites.

This technology opens up a new way to visualize changes in gene expression and may lead to the development of a whole new range of methods to study epigenetics. This is likely to have huge impacts in the way we study and treat complex or multifactorial diseases, enabling faster diagnosis and treatment and improving patients lives.

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ROSELLA: A FLUORESCENT PH BIOSENSOR FOR STUDYING AUTOPHAGY

By Beth Kenkel | April 5, 2017

Rosella is a pH-sensitive fluorescent biosensor that was recently deposited with Addgene by Dr. Mark Prescott. This system was developed for monitoring and analyzing autophagy of cytosol and organelles in yeast cells. Autophagy (Greek for "self-eating") is induced by a lack of nutrients and targets cytosol and organelles to the vacuole/lysosome for degradation and recycling. The key to Rosella's autophagy-sensing abilities is that its fluorescence emission spectrum changes when it goes from a more neutral pH compartment, like the cytosol, to the higher pH of the vacuole. Read on to learn more about prior methods for studying autophagy and how Rosella improves upon them.

Methods for Studying Autophagy

Biochemical Assays

- *Long-lived protein degradation*: This approach measures the degradation rate of radio-labeled long-lived proteins as a proxy for autophagy. Cells are cultured with isotope-labeled amino acids for several hours to several days, followed by a short period of growth with unlabeled amino acids. This washout period removes radio-labeled short-lived proteins primarily via proteasome degradation. Autophagy is then induced and quantified by measuring the amount of radioactivity in the culture supernatant (this is indicative of protein degradation). To control for protein degradation due to other pathways, it is standard practice to compare degradation rates between samples treated with or without an autophagy inhibitor. While this method does provide a quantitative measure of autophagy, it only measures bulk autophagy (as opposed to giving more fine-grained detail about what proteins or organelles are being degraded) and it is slow.

- *Alkaline phosphatase activity*: In yeast, the PHO8 gene encodes a vacuolar alkaline phosphatase. Normally PHO8 is synthesized at the ER, delivered to the vacuole via the secretory pathway, and then cleaved to generate an active form of the protein. To monitor autophagy, the Pho8Δ60 mutant protein is expressed. Pho8Δ60 localizes to the cytosol in an inactive form unless autophagy is induced. Then non-selective macroautophagy of the cytosol leads to accumulation of Pho8Δ60 in the vacuole, where it is cleaved to generate an active enzyme. Pho8 phosphatase activity or the molecular weight shift of Pho8 from its uncleaved to cleaved form is the final read out of this assay. This is also a quantitative measure of autophagy, but, like the protein degradation assay above, it's slow and only measures bulk autophagy.

Morphological Assays

- *Electron microscopy*: Electron microscopy is a traditional method for studying autophagy. This method relies on the identification of autophagic structures based on morphology. Autophagosomes are relatively easy to identify: double-membraned structures containing undigested cytoplasmic contents. Autophagosomes that have fused with the vacuole or a lysosome are trickier to identify because their contents can be at various stages of degradation. Additionally, electron microscopy analysis is time consuming.

- *GFP-tagged Atg8p or LC3*: ATG8 is part of a group of genes that affect autophagy (ATG) in yeast. Atg8p ("p" stands for protein) associates with autophagosomal membranes, so tagging it with GFP allows for tracking the localization or accumulation of pre-autophagosomal structures, autophagosomes and autophagic bodies in yeast. A GFP-tagged version of LC3, the mammalian Atg8p homolog, can also be used to monitor autophagy, but under some conditions it aggregates in an autophagy-independent

ROSELLA: A FLUORESCENT PH BIOSENSOR FOR STUDYING AUTOPHAGY (CONT'D)

manner. Additionally, tracking Atg8p or LC3 doesn't provide information about the contents of the autophagosome nor does it provide information about what is being degraded in the vacuole or lysosome, since both Atg8p and LC3 are degraded or released once an autophagosome fuses with the vacuole or lysosome.

Rosella Fluorescent Properties

Rosella is a dual color-emission biosensor named after the brightly-coloured Australian parrot. It's comprised of two tandem fluorescent proteins: a relatively pH-insensitive RFP variant, DsRed.T3 and a pH-sensitive GFP variant, super ecliptic pHluorin (SEP). They are connected by a 9 amino acid linker that's fused to the C-terminus of DsRed.T3. See Table 1 for a summary of Rosella's excitation and emission spectra. DsRed is the permanent fluorescent tag portion of Rosella: it will emit red fluorescence regardless of its localization in the cell. SEP's pH sensitivity means Rosella will fluoresce green unless it's in an acidic environment like the vacuole or lysosome. See Table 2 for a summary of what color fluorescence Rosella emits based on its localization.

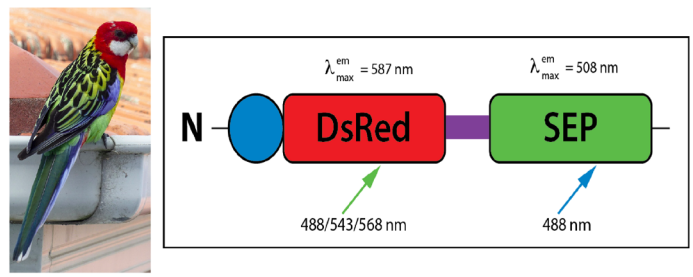

Figure 1. Left: Australian parrot from which the Rosella biosensor gets its name. Right: The Rosella biosensor. From the N-terminal end (N): targeting sequence for subcellular localization (not present in the cytosolic version of Rosella) - blue circle; DsRed.T3 - red box; 9 amino acid linker - purple bar; GFP-variant (SEP) - green box. Image designed by Jessica Welch.

Table 1: Summary of Rosella's excitation and emission spectra.

Component	Excitation	Emission	pH Range
DsRed. T3	488, 543*, 568 nm	587 nm	~4.9 - 9
SEP	488* nm	508 nm	~6.5 - 9

*Optimal sequential excitations wavelengths as determined by Rosado et al: see Table 2 for more details on the effect of pH on the fluorescent properties of Rosella.

Table 2: Effect of Localization on Rosella's fluorescence emission.

Localization	pH	Emission Color
Cytosol	~7.5	Red and Green
Mitochondria	~8	Red and Green
Vacuole	~6.2	Red
Lysosome	~4.8	Red

Using Rosella to Study Autophagy in Yeast

Rosella is targeted to the cytosol when expressed without a signal sequence and to the mitochondria via citrate synthase's mitochondrial targeting sequence. Under normal growth conditions, both variants are excluded from the vacuole and emit red and green fluorescence. After 4 hours of nitrogen starvation to induce autophagy, red but not green fluorescence accumulates in the vacuole. See Figure 2 for an example of what fluorescence looks like for cytoplasmic mitochondrial Rosella with and without autophagy. In both cases, red fluorescence in the

ROSELLA: A FLUORESCENT PH BIOSENSOR FOR STUDYING AUTOPHAGY (CONT'D)

vacuole increased with longer exposure to autophagy-inducing conditions.

Rosella makes it easier to study the mechanisms behind autophagy by tracking what's being transported to the yeast vacuole (i.e. cytosol, mitochondria), rather than tracking generic autophagy markers. It's particularly useful for comparing bulk autophagy vs. mitophagy, the targeted autophagy of mitochondria. As shown in Sargsyan et al. It is also possible to make Rosella fusions and thereby monitor autophagy of your protein of interest.

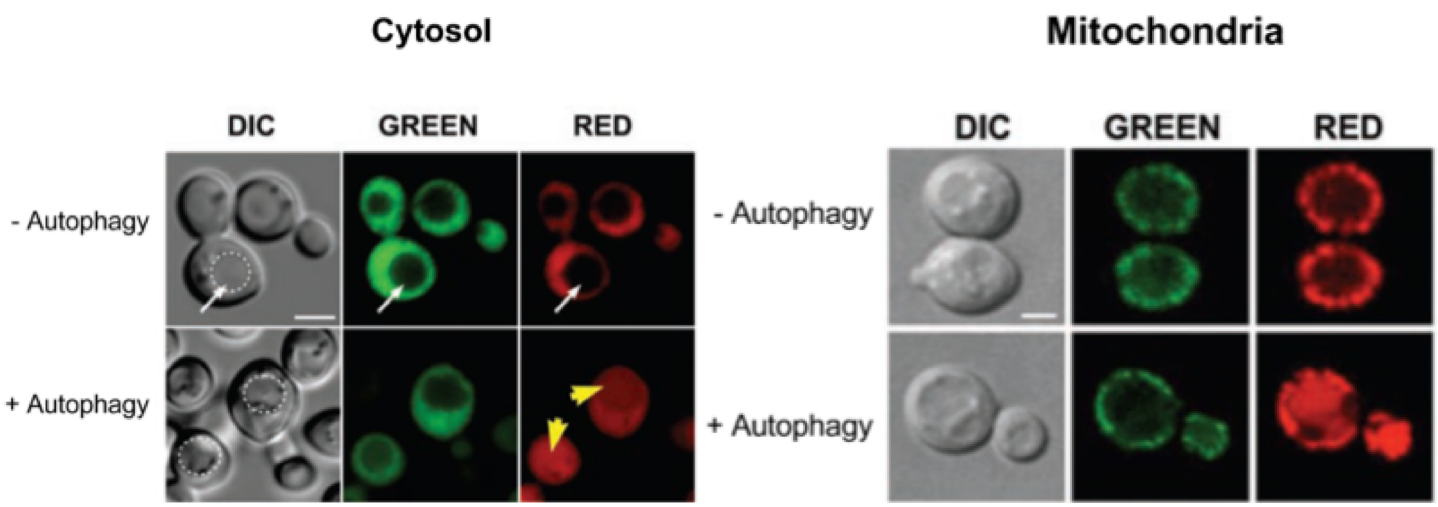

Figure 2: Rosella expressed in yeast cytosol (left) and mitochondria (right) is delivered to the vacuole following nitrogen starvation. White arrows and dotted lines: empty vacuoles that lack fluorescence. Yellow arrows: vacuoles emitting red fluorescence. Modified from Rosado et al.

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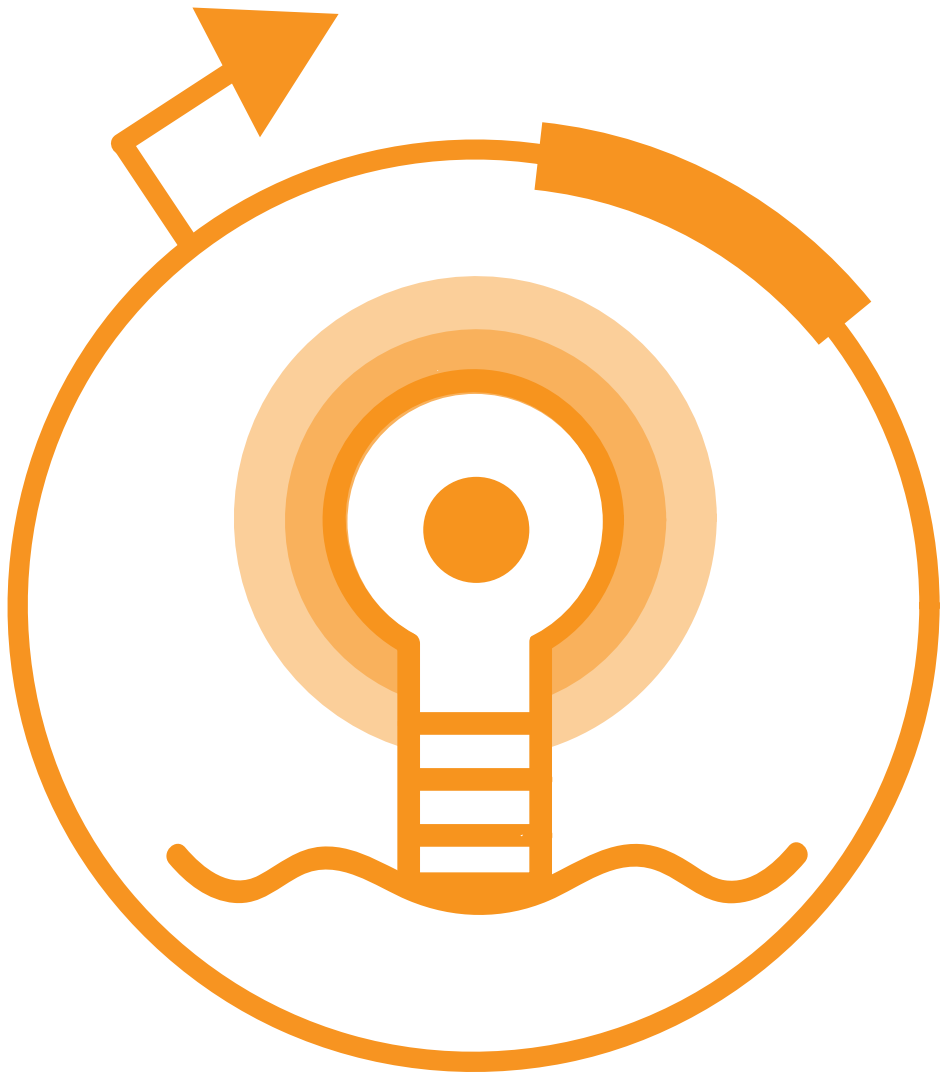
CHAPTER 7: NON-PROTEIN FLUOROPHORES



BETTER DYEING THROUGH CHEMISTRY & SMALL MOLECULE FLUOROPHORES

By Luke Lavis | September 8, 2016

Chemistry is Dead, Long Live Chemistry

The discovery of green fluorescent protein (GFP) sparked a renaissance in biological imaging. Suddenly, cell biologists were no longer beholden to chemists and (expensive) synthetic fluorophores. Add a dash of DNA with an electrical jolt and cells become perfectly capable of synthesizing fluorophore fusions on their own. Subsequent advances in fluorescent proteins have replicated many of the properties once exclusive to small-molecules: red-shifted spectra, ion sensitivity, photoactivation, etc. These impressive advances lead to an obvious question: In this age of GFP and its ilk, why should cell biologists talk to chemists?

One reason is something called “the photon budget”: each sample has a limited number of fluorophores and each fluorophore can emit a limited number of photons before bleaching. The amount of information one can extract from a biological sample is wholly dependent on the photon budget and pushing the frontiers of fluorescence microscopy often requires more photons. For example, moving from transient overexpression of protein fusions to gene-edited cells can decrease the number of fluorophores, compromising the photon budget. Likewise, the switch from ensemble imaging to single-molecule imaging places a greater burden on the photon budget, which determines how long and precise we can track individual molecules. Poor photon budgets are a widespread issue with fluorescent proteins—even the thriftiest cell biologist can feel like a college student searching couch cushions for spare change, desperate to extract a few more photons from a sample.

Chemical fluorophores can be substantially brighter and more photostable than fluorescent proteins, providing a straightforward way to improve the photon budget. Of course, “reverting” to small molecule dyes seems daunting—no one wants to spend their days microinjecting fluorescent conjugates into cells. Fortunately, over the last 20 years clever chemists and biochemists have developed techniques to make labeling chemistry easier and more functional in complex biological environments such as live cells and tissues (Figure 1). These flexible strategies give you the best of both worlds: the excellent photophysics of chemical dyes combined with the ease and specificity of fluorescent proteins.

In-cell Labeling Strategies

The majority of in-cell labeling strategies have two parts: (1) a genetically encoded “tag” expressed as a fusion with your favorite protein and (2) a synthetic fluorophore-containing “ligand” that binds to the tag. Like most good ideas in biological imaging, the initial breakthrough for in-cell labeling techniques was provided by Roger Tsien, who showed that the selective interaction between a bisarsenical dye ligand (e.g., FlAsH, ReAsH) and a short genetically encoded tetracysteine (Cys4) peptide tag could be used to label proteins in cells (Figure 1a). Other strategies that have been developed based off of this concept include:

- Self-labeling tags (e.g., SNAP-tag, HaloTag, TMP-tag) – These widely used systems consist of a genetically encoded enzyme variant tag that reacts specifically and irreversibly with a small substrate ligand motif attached to a fluorophore (Figure 1b).
- Engineered ligases (e.g., lipoic acid ligase, biotin ligase, phosphopantetheinyltransferase) – These enzymes catalyze the covalent attachment of a fluorophore ligand to a peptide tag (Figure 1c).
- Click chemistry (e.g., transcyclooctene–tetrazine)– Nonnatural amino acids can be incorporated into a protein structure and then used with the growing toolbox of bioorthogonal chemical reactions (i.e., click chemistry) to attach a fluorophore at the specific site of incorporation (Figure 1d).
- Fluorogen activating proteins (FAPs) – These modified antibody fragments bind and enhance small molecule fluorogens (Figure 1e).
- Stains – These labels consist of a fluorophore conjugated to molecular species with high affinity for

BETTER DYEING THROUGH CHEMISTRY & SMALL MOLECULE FLUOROPHORES (CONT'D)

endogenous molecular targets such as paclitaxel. Unlike other systems, no genetically encoded tag is required as the small-molecule binding motif targets endogenous protein (Figure 1f).

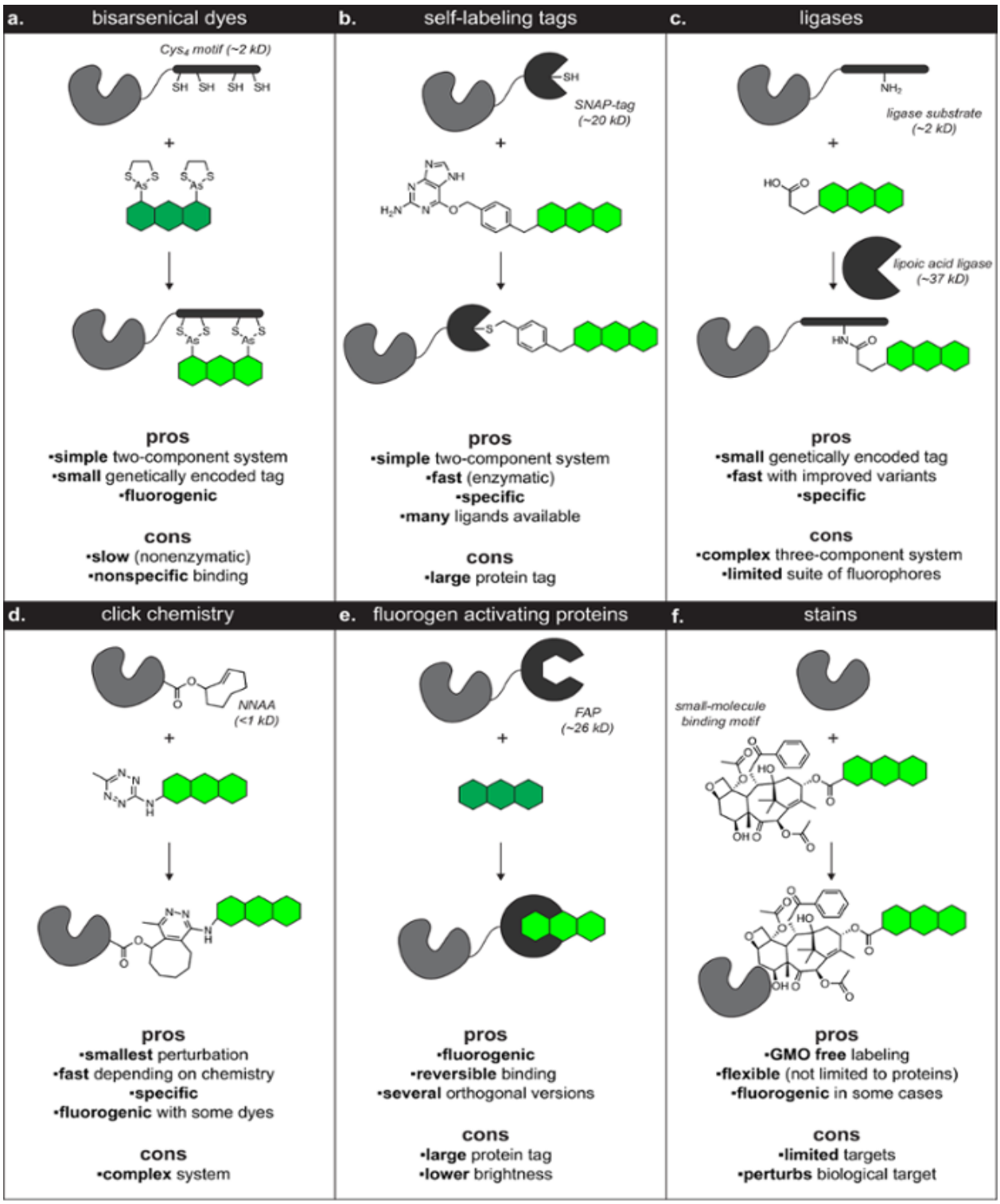


Figure 1. Live-cell labelling strategies using small-molecule fluorophores. In all cases, the green structure represents the fluorophore while the gray shape is the protein of interest.

BETTER DYEING THROUGH CHEMISTRY & SMALL MOLECULE FLUOROPHORES (CONT'D)

Labeling Strategy Pros and Cons

All of these labeling strategies have trade-offs between the size of the genetically encoded tag, the speed and selectivity of the fluorophore attachment, the brightness of the resulting conjugate, and the complexity of the system. A particularly desirable property for small-molecule labeling strategies is fluorogenicity, meaning the ligand exhibits low fluorescence when free in solution, but high fluorescence upon binding to its cognate protein. In some cases this property eliminates the need for removing excess dye from the sample, which is particularly important for samples where washing is difficult, such as intact tissue. Some chemical labeling strategies such as the bisarsenical dye (Figure 1a) and FAP systems (Figure 1e) are inherently fluorogenic and several others can be made fluorogenic using environmentally sensitive fluorophores. Overall, self-labeling tag systems (Figure 1b) are perhaps the best method for live cell labeling and the easiest switch from fluorescent proteins given the relative simplicity of the system and the availability of fluorescent and fluorogenic ligands.

New Fluorophores

As a corollary to these innovative labeling strategies, several groups—including mine—have been revisiting the old chemistry of dyes. The first synthetic dye, mauvine, was discovered in 1856 by William Perkin. His discovery set off a flurry of activity and the majority of the classic small molecule fluorophores, such as rhodamines (Figure 2a), were discovered in the 19th century. Many of the small-molecule labeling techniques have focused on this classic, net-neutral dye scaffold due to its established chemistry, small size, brightness, and cell permeability. In past decades, further refinements to this established dye structure have yielded commercial panels of advanced rhodamine fluorophores, such as the Alexa Fluor and ATTO dyes, with improved brightness, photostability, and spectral range. Nevertheless, these fluorophores were designed as antibody and oligonucleotide labels for fixed-cell imaging, not for live-cell applications. Thus, these fluorophores are often bulky and contain polar groups (Figure 2b), which can preclude their use inside living cells. We recently discovered that incorporation of four-membered azetidine rings could substantially enhance the brightness and photostability of the classic rhodamines and their analogs without sacrificing their small size and membrane permeability (Figure 2c). These cell-permeable Janelia Fluor (JF) dyes have excellent properties inside living cells, especially for advanced imaging experiments. We continue to develop derivatives that have different spectral properties, exhibit high fluorogenicity, are photoactivatable, and function *in vivo*.*

Final Thoughts

The ease of use and continual improvement of fluorescent proteins makes them the go-to choice for many (if not most) fluorescence microscopy experiments. However, when your budget gets a bit thin, small-molecule labeling approaches can provide a fresh infusion of photons for your imaging experiment. Innovative labeling strategies and improved fluorophores are making chemical dyes increasingly attractive and accessible to cell biologists—and we are not done yet. Further refinements to these systems—smaller tags, brighter conjugates, higher fluorogenicity, photoactivation etc.—can further increase the photon budget and allow us to push further the frontiers of biological imaging.

*Worried about your budget? Email janeliafluor@janelia.hhmi.org to try out the JF dyes.

Check Out Janelia's New Institution Page at Addgene.

BETTER DYEING THROUGH CHEMISTRY & SMALL MOLECULE FLUOROPHORES (CONT'D)

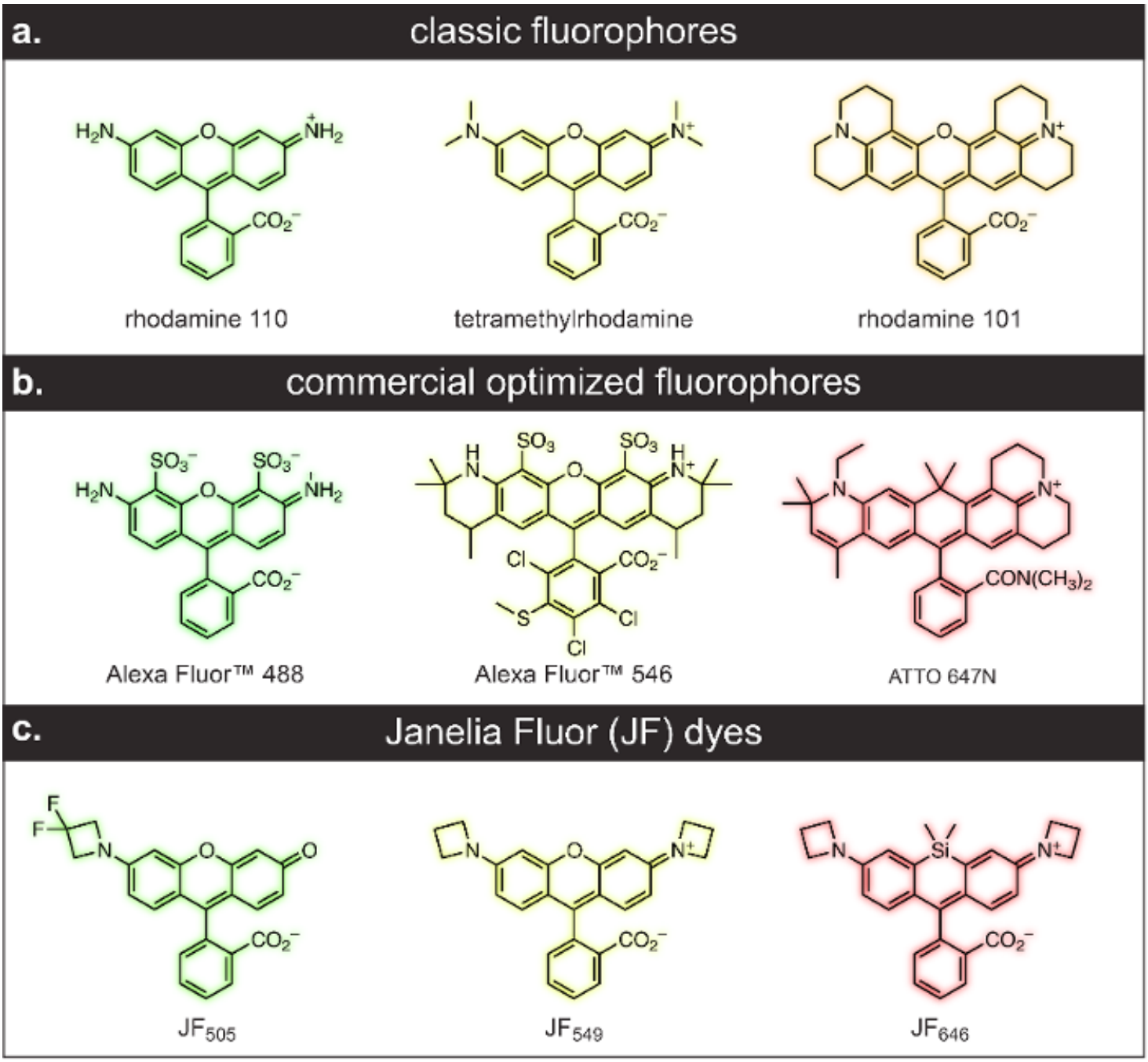


Figure 2. Small-molecule fluorophores used in biological imaging. Color indicates the emission maximum of the fluorophore.

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APTAMER FLUOROPHORES

By Eric J. Perkins | April 11, 2017

What is an Aptamer?

Nearly 30 years ago, two independent groups, led by Jack Szostak and Larry Gold, developed methods for selecting and amplifying RNA sequences that could bind very specifically to target molecules. Using a technique called systematic evolution of ligands by exponential enrichment (SELEX), some 10^{10} oligonucleotides could be screened for their affinity to a wide range of non-nucleotide targets. These RNA molecules, which could bind their targets with high specificity and affinity, were eventually called aptamers, from the Latin aptus, meaning "to fit". SELEX could be used to classify DNA aptamers as well, and over the course of the next two decades, these nucleotide-based ligand binders would prove to be highly adaptable tools.

The key to aptamers' flexibility is their broad range of targets, including proteins, peptides, amino acids, drugs, metal ions, and cells (even pathogenic bacteria). That range is possible because aptamers can either fold to incorporate their target molecule, such as a metal ion, or fold to incorporate themselves into a larger target, such as a protein. Such flexibility makes aptamers ideal candidates for biosensors, which are used in a variety of applications, including detection of environmental pollutants, drug discovery, and disease diagnosis.

As biosensors, aptamers hold a few important advantages over their amino acid-based counterparts, antibodies and enzymes, such as:

1. Design and selection for targets can be done largely *in vitro*;
2. Easy and reproducible commercial synthesis (those of us who have used antibodies for diagnostics know how frustrating it can be to get a "bad batch");
3. Customizable sensitivity and detection - since they frequently undergo conformational changes after target binding, aptamers are highly customizable in terms of how they actually "report" as biosensors.

Making Aptamers Glow

The flexibility of aptamers means they can be used with or as fluorophores, in several different ways. Researchers have used oligonucleotides in fluorescent visualization techniques for decades, most notably for fluorescent in situ hybridization (FISH) where fluorophore-linked antibodies are used to visualize RNA. Low signal-to-noise ratios with such techniques can be problematic, however. Well designed aptamers can help reduce such background, thereby improving traditional visualization techniques. In addition, their incredible versatility opens aptamers for use in many entirely new applications.

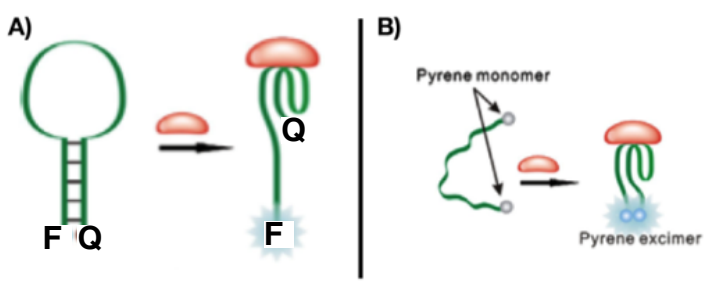

Figure 1. (A) A fluorophore (F) and quencher (Q) are in close proximity when the aptamer is in its unbound stem loop structure, and that secondary structure is disrupted upon ligand binding, activating the fluor. (B) If the fluorophore is dimerized, ligand binding can also unite the monomers to activate fluorescence. Adapted from Song, 2008.

Aptabeacons

Fluorescence resonance energy transfer (FRET) technology has been studied and used in assays for over half a century; the Addgene blog has an excellent primer and tips from experts on FRET techniques. By combining standard donor/acceptor FRET know-how with aptamers, aptabeacons (or aptatags) were born. Aptabeacons

APTAMER FLUOROPHORES (CONT'D)

can be created in many different ways, but the general premise is that an aptamer in its unbound state contains a fluorophore and a quencher in close proximity. For instance, the fluorophore and quencher may form the base of the stem in an RNA stem-loop structure (see Fig. 1a). When the aptamer binds its ligand, the stem loop is disrupted, removing the quencher from the proximity of the fluorophore and subsequently leading to a positive signal. The same effect can be achieved for a fluorophore that relies on dimerization for activation, only in this case ligand binding would bring the relevant ends together rather than separate them (Fig. 1b). One can also make "signal-off" biosensors in which the aptamers fluoresce until they bind to the appropriate ligand, but relying on a lack of fluorescence for readout may render this approach less sensitive in most applications.

Light-up Aptamers (or, Fluorescing with Veggies)

Though the aptabeacons can be used as powerful biosensors, the bound protein fluorophores and quenchers are still relatively bulky. One of the advantages of oligonucleotides over proteins is their modularity, which is lost as soon as the aptamer is bound to a protein fluor. Transcribing and translating a protein fluorophore also takes time - bypassing translation altogether with an oligonucleotide-only sensor could save precious minutes in a time sensitive detection assay.

In 1999, Grate and Wilson paired laser cleavage and an RNA aptamer that bound the compound malachite green (MG) to create a non-protein based visualization technique. Ultimately, MG proved to be too toxic for many *in vivo* applications, but this work proved the concept that RNA could be used to induce fluorescence outside the context of a paired fluorophore protein. Years later, Samie Jaffrey's laboratory took advantage of their deep knowledge of the nature of GFP fluorescence to create an improved fluorescent aptamer system. Knowing that GFP's structure stabilizes a 4-hydroxybenzylidene imidazolinone (HBI)-like compound allowing it to fluoresce, the Jaffrey lab asked: Could an RNA aptamer do the same thing?

Using the HBI derivative 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) with relatively low fluorescence *in vivo*, the Jaffrey lab used SELEX to find a 98 nt RNA aptamer that would bind the fluorogen and increase its fluorescence, essentially mimicking GFP. Though the aptamer, called Spinach, required longer exposure time than its protein equivalent and was less stable due its RNA nature, it paved the way to new biosensor possibilities. For instance, further selection of Spinach with a target metabolite creates an aptamer that will only bind to DFHBI in the presence of that metabolite (Fig.2).

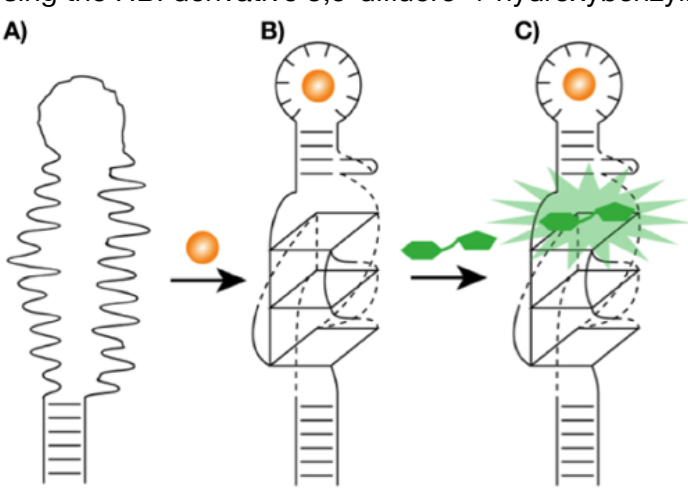

Figure 2. (A) A misfolded RNA aptamer binds to its target metabolite ligand, represented by the orange sphere. (B) This binding promotes proper binding of its own stem, which in turn allows the aptamer to stabilize a fluorogen. (C) Once stabilized, the fluorogen lights up. Adapted from Ouellet, 2016.

Spinach eventually evolved into Spinach2, a more stable and brighter version of the aptamer, but it still had limitations *in vivo*. Using a combination of SELEX and fluorescence-activated cell sorting (FACS) of *E. coli* transformed with aptamer libraries, the Jaffrey group selected for a 49-nucleotide aptamer they called Broccoli. As a product of SELEX and FACS, Broccoli is inherently more stable in cells. The new aptamer also has a higher melting temperature than its Spinach ancestors, and has a lower magnesium dependence. Though initially still unstable in eukaryotes, the Jaffrey lab has since developed new RNA-based scaffolds to contain

APTAMER FLUOROPHORES (CONT'D)

and stabilize Broccoli cassettes, thereby extending their use into mammalian cells. They've also dimerized Broccoli to further enhance its biosensor capabilities (see Fig. 1b).

The Future Looks Bright

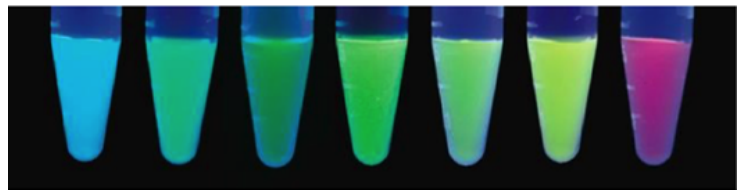

Figure 3. An array of aptamer fluors. Photo courtesy of the Jaffrey Lab.

The Jaffrey lab and others continue to hone their selection techniques to come up with new colors (Fig. 3), such as Radish and Carrot, and further means to increase aptamer stability and decrease background in vivo. The nature of aptamers makes them excellent candidates for developing assays for otherwise difficult-to-study RNA-modifying enzymes, such as the RNA demethylases FTO and ALKBH5. Their ease of production have also made aptamers an important component of high throughput assays with microarrays and biochips. Ultimately, there are just some applications that lend themselves to aptamer-based instead of protein-based fluorophores, including:

- Environments with higher temperatures, since proteins can denature while aptamers are more stable and can go through cycles of denaturation/renaturation.
- *In vivo* assays in which protein fluors are likely to trigger immunoresponses. Nucleotides, in general, are far less immunogenic than proteins.
- Assays that require recognition of ions or particularly small molecules that do not generate an immune response. Aptamers can be selected for these small ligands.

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APTAMER FLUOROPHORES (CONT'D)

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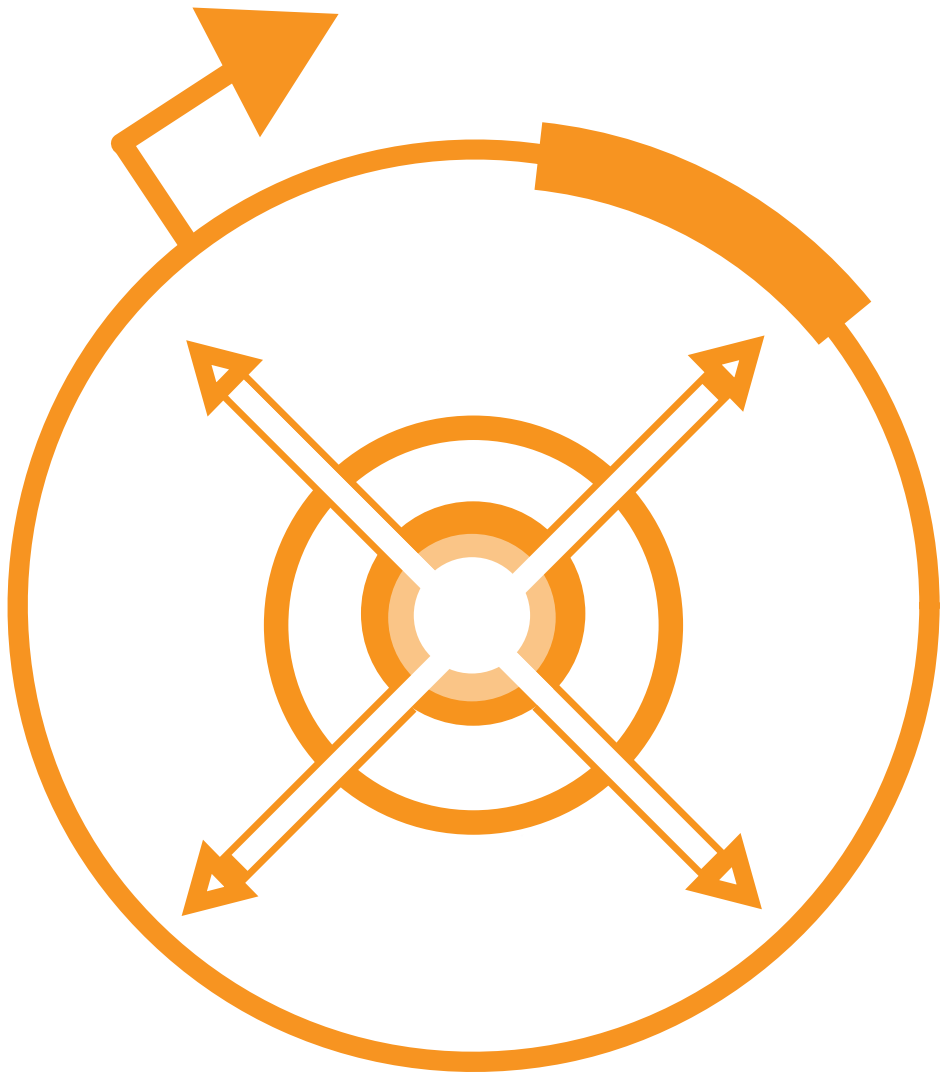
CHAPTER 8: OTHER APPLICATIONS OF FLUORESCENT PROTEINS



CONTROLLING PROTEIN ACTIVITY WITH GFP

By Mary Gearing | November 24, 2015

At Addgene, we love GFP, and we're always excited when depositors find new ways to make this workhorse protein even more useful! From FPs optimized for oxidizing environments to photoconvertible variants, it seems like GFP is always learning new things. Now, work from Connie Cepko's lab allow researchers to activate transcription or Cre recombinase activity only in the presence of GFP. These systems, known as T-DDOG and Cre-DOG, respectively, repurpose popular GFP reporter lines for more sophisticated experimental manipulations, saving the time and money needed to develop new lines.

Using Nanobodies to Create GFP Scaffolds for Transcriptional Activation

The project began with a frustration many researchers have experienced: wanting to ask and answer experimental questions, but not having the tools needed to do so. Cepko lab researcher Jonathan Tang wanted to express a variety of proteins in single cell types in the mouse, but knew it would take years to develop the mouse lines required for this purpose. Tang wondered if he could use previously established mouse lines expressing GFP in specific cell types to do more than just label cells. If GFP could be co-opted to control gene expression, he could then selectively manipulate only GFP-labeled cells.

Once Tang and Cepko found a description of GFP-binding nanobodies, the project really took off. Unlike most antibodies, nanobodies, which occur naturally in camels, consist of a single heavy chain, and they are small and stable inside cells. Nanobodies binding GFP were designed in 2009. Subsequent work showed that nanobodies could be fused to other proteins, and these fusions retained the ability to bind GFP.

The idea of using GFP as a scaffold suddenly seemed very realistic. Envisioning GFP as a substrate promoting dimerization, Tang et al. tested pairs of their GFP-binding proteins (GBPs) to find those that could co-occupy GFP. Once they'd found suitable pairs, they constructed three components: GBPa-VP16 (activation domain); GBPb-GAL4 (DNA-binding domain); and a UAS-driven luciferase reporter construct. In the absence of GFP, no reporter output was observed in their 293 cell culture system. In cells cotransfected with GFP and the GBPs, GFP linked the two GBPs together to create a complete transcription factor, activating luciferase transcription. Tang et al. coined this system T-DDOG (Transcriptional Devices Dependent On GFP).

In testing the specificity of their system, Tang et al. found that YFP and CFP can activate certain T-DDOG constructs similarly to GFP, but commonly used red fluorescent proteins dsRed, mCherry, and TdTomato do not induce transcription. Thus, T-DDOGs can be used in combination with green-red Cre-lox systems. T-DDOGs can also be designed with other DNA-binding domains, including the commonly used LexA and rTetR systems such that they activate gene expression from different promoters.

Moving to an *in vivo* system, Tang et al. electroporated T-DDOGs, GFP, and a reporter construct into the mouse retina. GFP expression robustly activated the reporter gene TdTomato, whose expression was absent without electroporated GFP. They then tested T-DDOGs with two mouse GFP reporter lines; again, TdTomato was seen only in cells with GFP, at a high activation frequency of 56-93%. In the converse test, 98% of TdTomato expressing cells were GFP+, indicating a robust, but specific system. T-DDOGs also successfully regulated expression of channelrhodopsin-2, commonly used in optogenetics, opening up the possibility of adapting GFP lines for optogenetics experiments in specific cell populations.

CONTROLLING PROTEIN ACTIVITY WITH GFP (CONT'D)

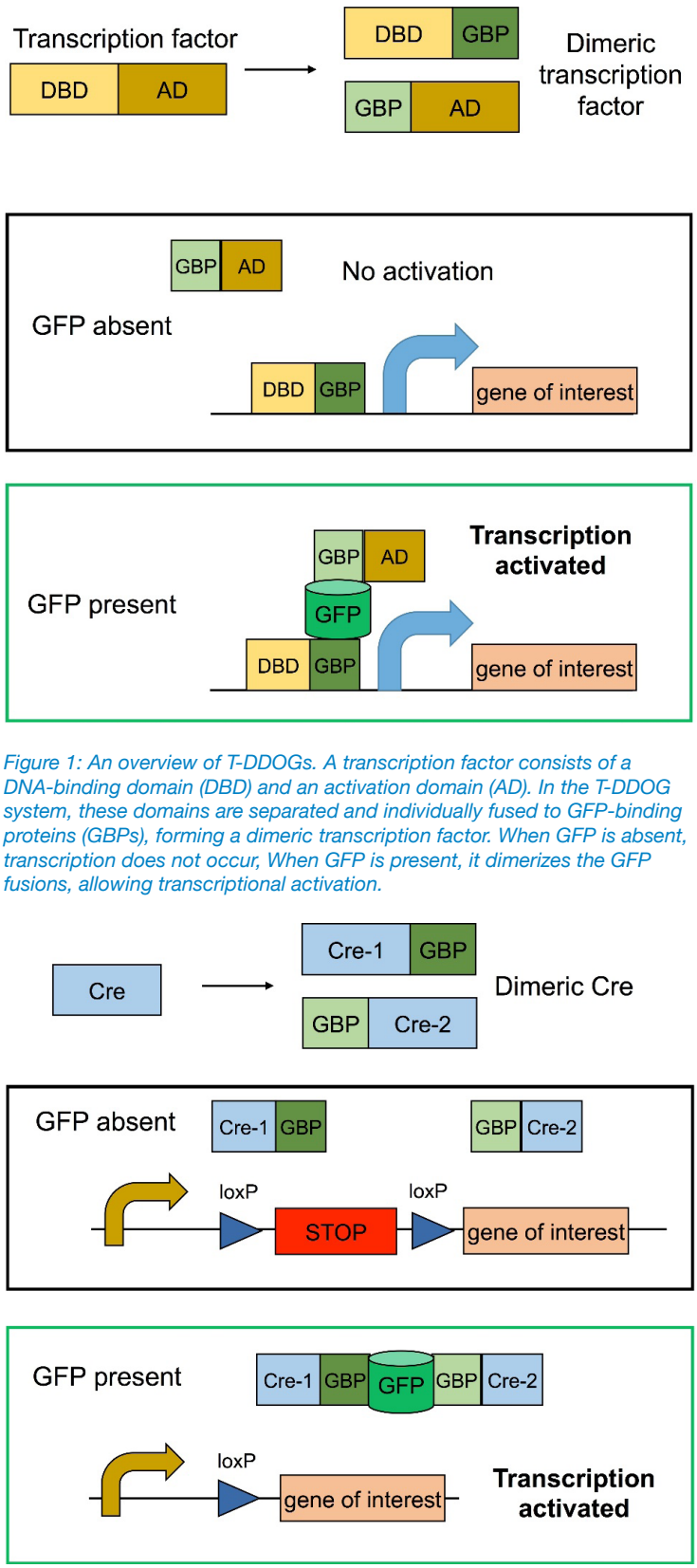


Figure 1: An overview of T-DDOGs. A transcription factor consists of a DNA-binding domain (DBD) and an activation domain (AD). In the T-DDOG system, these domains are separated and individually fused to GFP-binding proteins (GBPs), forming a dimeric transcription factor. When GFP is absent, transcription does not occur, When GFP is present, it dimerizes the GFP fusions, allowing transcriptional activation.

Combining Cre and GFP with Cre-DOGs

The T-DDOG results showed that GFP could regulate transcription in a cell-type specific manner, and Tang and Cepko were eager to see if they could apply GFP regulation to other types of proteins. They started with another biological workhorse, Cre recombinase, which induces recombination at LoxP sites. Placing a lox-STOP-lox cassette upstream of a gene of interest blocks transcription when Cre is absent. When Cre is present, it removes the STOP cassette, activating gene expression.

Although Cre isn't a modular protein, Tang et. al were able to create a split version of Cre that is only active when dimerized by the GBPs/GFP. The new system, CRE-DOG (Cre Dependent On GFP), is activated by GFP and derivatives GFP and YFP, but not red fluorescent proteins, as seen with T-DDOGs.

Like T-DDOGs, Cre-DOG is both robust and specific. When tested in retinal electroporation studies, DsRed reporter expression was induced in ~76% of GFP+ cells, and 100% of DsRed+ cells also expressed GFP. Using AAV constructs, Tang et al. found that Cre-DOG could be delivered throughout the nervous system, including the motor cortex, cerebellum, and spinal cord. Cre-DOG is also suitable for optogenetic studies, and since cells infected with AAV-Cre-DOG retain normal neuronal function, this system may make optogenetics in specific cell populations even easier.

Figure 2: An example application of Cre-Dog. Cre is split to create a dimer, with each portion fused to a GFP-binding protein (GBP). When GFP is present, Cre is activated and can induce recombination of a lox-STOP-lox cassette, promoting transcription of a gene of interest.

CONTROLLING PROTEIN ACTIVITY WITH GFP (CONT'D)

Advantages and Other Possibilities

It's clear that Cre-DOG and T-DOGGs open up many new possibilities for GFP-labeled lines, and they each have their own advantages. T-DOGGs can be made drug-inducible using the rTetR system, but Cre-DOG lacks the toxicity that can be seen with high levels of transcription activation domains. Both systems are easily adaptable to neuroscience applications, including optogenetics, and they should make it much easier to conduct functional studies in the nervous system.

More broadly, Cepko and Tang note that their scaffold system is likely applicable to many types of proteins. For proteins with a modular structure, constructing split variants should be relatively straightforward, but it is also possible to create split versions of non-modular proteins like Cre. Could Cas9-DOG be developed soon to allow genome modification only in a small subset of cells? Other proteins may also be utliized as scaffolds, including the set of red fluorescent proteins.

The T-DOGGs and Cre-DOG are available from Addgene, and we're eager to see how you use them in your research!

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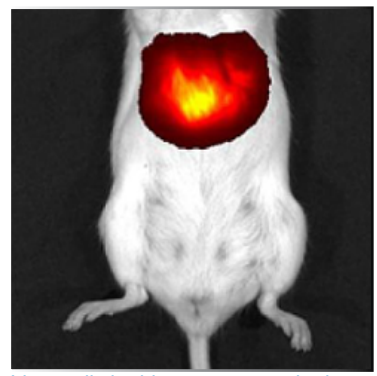
IN LIVING COLOR: THE SKINNY ON IN VIVO IMAGING TOOLS

By Kendall Morgan | Mar 27, 2014

If you start poking around on Addgene's Fluorescent Protein Guide to *In Vivo* Imaging, you'll pretty quickly notice the name Vladislav Verkhusha popping up again and again, and for good reason.



Liver cells in this mouse contain the fluorescent protein iRFP. Image credit: Albert Einstein College of Medicine.

We all know scientists have used fluorescent proteins to observe what's happening inside cells for at least a couple of decades. Green is the classic color, but fluorescent proteins are available in a variety of hues. While those tools are great for many applications, Verkhusha and his lab at Albert Einstein College of Medicine in New York recognized their limitations for peering right through living animals to see their organs – a liver or brain, say, or maybe a tumor. They wanted to find something better.

Transparency Window

That's exactly what they reported doing in a paper that appeared in Nature Biotechnology back in 2011 and then in another Nature Methods paper in 2013. The key to their bacterial phytochrome-derived proteins, iRFP670, iRFP682, iRFP702, iRFP713 and iRFP720, is that they absorb and emit light in the near-infrared portion of the electromagnetic spectrum – the spectral region in which mammalian tissues are nearly transparent.

As Verkhusha explained it to me, the transparency window of mammalian tissues is set by the properties of hemoglobin and melanin pigments, which absorb the majority of light below 650 nanometers (nm), and the absorbance of water, which absorbs wavelengths above 900 nm or so. In other words, wavelengths between 650 nm and 900 nm will pass through animal and human tissues largely unimpeded.

The challenge then was to develop fluorescent proteins that would fall within that near-infrared range, and that's exactly what iRFPs achieved. Those proteins allowed a signal-to-background ratio in mammalian tissues 20-fold greater than any fluorescent protein earlier described.

"Until our proteins, there were lots of fluorescent proteins made from jellyfishes and corals, but all of them fluoresce outside the transparency window of mammalian tissues," Verkhusha said. "We developed near-infrared fluorescent proteins within this transparency window, so now, we could see deeper."

Adding to the Toolbox

Verkhusha added two far-red light photoactivatable (PA) near-infrared fluorescent proteins (FPs), called PAiRFP1 and PAiRFP2, to the *in vivo* imaging toolbox, which increase their fluorescence upon illumination with far-red light. As the researchers described in Nature Communications last year, "The capability to control spectral properties of PA FPs with light of specific wavelength and intensity allows for optical labeling and tracking of proteins, organelles, and living cells in a spatiotemporal manner, which is not possible with conventional FPs. In addition, PA FPs can improve the achievable signal-to-background ratio, thus, allowing higher resolution in samples containing substantial autofluorescence background."

In later work in Scientific Reports, Verkhusha's team reported another advance in the application of near-infrared fluorescent proteins, iRFP670 and iRFP720, as photoacoustic contrast agents for two-color imaging in animals. The approach relies on ultrasound waves as opposed to light to produce higher resolution images *in*

IN LIVING COLOR: THE SKINNY ON IN VIVO IMAGING TOOLS (CONT'D)

vivo.

"One of the reasons we can't localize one cell at a depth of one centimeter with fluorescence is because the light becomes very scattered," Verkhusha explained. Ultrasound wavelengths, on the other hand, are larger than the size of a cell, so they scatter much less. As a result, ultrasound enables higher resolution images at depths up to eight millimeters – not single cells (yet) but small clusters of cells.

Looking Ahead

What's next, you might wonder? Verkhusha says they are always looking to improve upon existing genetically-encoded probes by making them brighter, more photostable, smaller, and less toxic to cells. They'd like to make near-infrared fluorescent proteins that operate within the 750 to 800 nm range, and his team is also looking in the direction of biosensors capable of revealing, for example, how muscles work inside a living animal.

For those making use of these probes, he says, when in doubt, read the methods sections of the relevant papers, and then read them again. Most of the time, the answers will be there.

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BRAINBOW

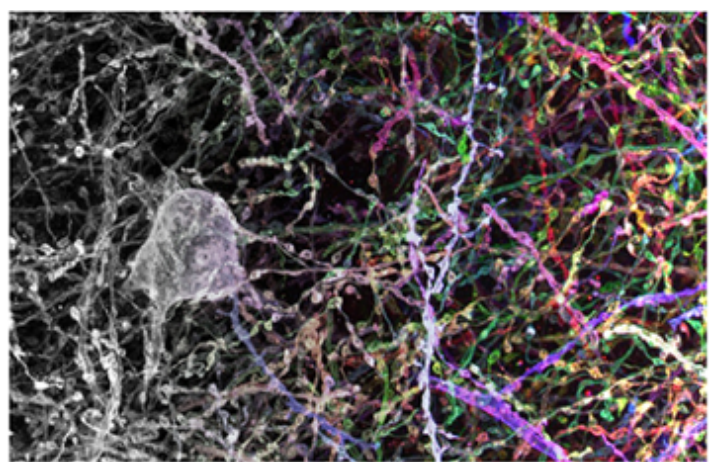
By Mary Gearing | April 24, 2015

CRISPR-Cas9 genome editing may be the hot new way to manipulate gene expression, but other gene manipulation systems remain valuable to biology. Cre-lox recombination, discovered in the 1980s, is one of the most important ways to spatially and temporally control gene expression, especially in *in vivo* models, and new Cre-lox based technologies are still being developed today. In this post, I will highlight the evolution of the Brainbow multicolor labeling system - a perfect example of the continued utility of Cre-lox. Check out our blog post, Plasmids 101: Cre-lox, if you need a quick primer on how Cre-lox recombination works.

Advances in Cre-lox recombination have included inducible Cre, such as the tamoxifen-responsive CreERT, the coupling of Cre-lox with FLP-FRT recombination to generate additional recombination events, and the synthesis of alternative loxP sites to specify distinct patterns of recombination. In 2007, Joshua Sanes and Jeff Lichtman, both of Harvard's Department of Molecular and Cellular Biology, harnessed these attributes of Cre-lox to create the Brainbow mouse neural labeling system. Brainbow-1 and -2 have been further refined to create Brainbow 3.2. Brainbow labeling has also been applied to cell marking and lineage tracing in other systems, such as zebrafish and *Drosophila*.

Why Brainbow?

Brainbow highlights the trend in biology towards single-cell resolution imaging. Sanes and Lichtman endeavored to create a map of the brain, a connectome that would detail how neurons form circuits and where they synapse. See the overview schematic at the bottom of this post for a basic synopsis of the differences amongst the Brainbow systems. Pre-Brainbow techniques, such as Golgi staining, injection of diffusible labels, or electron microscopy of stained sections, were limited due to low resolution or were very time-intensive, necessitating the development of a new technique. In a structure as tightly packed and interwoven as the brain, robust single-cell labeling is necessary to distinguish individual cells.



AAV-Brainbow labeled hippocampal interneuron axons. Image courtesy of Dawen Cai.

The Brainbow-1.0 construct contains three fluorescent proteins: RFP (red), YFP (yellow) and M-CFP (membrane tethered cyan). Without Cre recombination, RFP is expressed. Cre can mediate one of two deletions to allow YFP or M-CFP expression; these deletions are defined using 2 loxP variants (only identical loxP sites can mediate recombination.) The deletions are mutually exclusive, as either deletion removes one of the other loxP site variants, preventing further recombination. Brainbow-1.1 scales up the system to four fluorescent proteins, with recombination events specified by three loxP variants, but the principles remain the same. The first fluorescent protein in the construct, OFP (orange), is expressed when Cre is absent; stochastic Cre excision results in one of three recombinations, and subsequent recombinations cannot occur.

While the Brainbow-1 system employs Cre-mediated deletion, the design of Brainbow-2.1 cleverly combines Cre-mediated deletion and inversion. The Brainbow-2.1 construct can express one of four colors (n-GFP, RFP, YFP or M-CFP.) The construct contains two tandem invertible segments, each with two fluorescent protein coding sequences in opposite orientations. Cre-mediated excision removes one segment, eliminating two color

BRAINBOW (CONT'D)

possibilities. The remaining cassette can invert as long as Cre remains expressed; once Cre is removed from the system, the fluorescent protein whose coding sequence is closest to the promoter will be expressed. This system differs from Brainbow-1.0 in that transient Cre recombination is required to prevent constant inversion of the construct.

Sanes and Lichtman used pronuclear injection to insert these Brainbow constructs into mice. When they imaged the mouse brains, they found many more than 3-4 colors due to the tandem integration of multiple copies of the construct (about 8 in Brainbow-1.0 mice.) The combinatorial effect of multiple, independent recombination events leads to a rainbow of colors. With three copies of the construct, one would expect ten colors (see table below); in various reports, Sanes and Lichtman have observed from 90-160 distinct colors due to higher copy number. The name Brainbow is truly fitting for this colorful technology.

Optimizing the System: Brainbow-3

Although Brainbow provided neuroscientists with the vast array of colors needed to mark individual neurons, the system also suffered from a number of limitations. First, the imaging of Brainbow mouse tissue was challenging due to low fluorescence intensity, caused partially by fluorophore photoinstability, as well as the tendency of fluorophores to aggregate in the neurons' somata. Second, the system did not permit analysis by immunostaining. Although the fluorophores used are fluorescently distinct, their protein sequences are highly homologous, preventing design of antibodies specific to each fluorophore. Third, Brainbow-1 and Brainbow-2 each contained a "default" state; for example, Brainbow-1.0 expresses RFP when the construct has not undergone recombination. This default state was disproportionately expressed by a majority of neurons, limiting the number of distinct colors that could be observed in a given area.

In 2013, Dawen Cai, et al. released a refinement of the Brainbow technology, Brainbow-3.0, with the goal of overcoming the limitations listed above. First, they screened a variety of fluorescent proteins to find those with ideal characteristics (low aggregation, high photostability, and high stability after fixation). From the seven resulting proteins, they chose three with low levels of both fluorescence and sequence overlap (coral mOrange2, jellyfish EGFP, and sea anemone mKate2). They then successfully generated custom antibodies to each of these proteins and confirmed that there was no cross-reactivity, opening the door for immunostaining analysis. To achieve even cell labeling, they generated farnesylated derivatives of the fluorescent proteins, which are directly trafficked to cell membranes. Membrane trafficking of farnesylated derivatives enables labeling of delicate axonal and dendritic processes not previously visible with Brainbow-1 and -2.

The general structure of Brainbow-1.0 is retained in Brainbow-3.0, but with mOrange2, EGFP and mKate2 as the fluorophores; mOrange2 is the default state. In contrast, Brainbow-3.1 and -3.2 do not display default fluorescence due to the addition of translation-blocking STOP cassette immediately following the promoter. The STOP cassette includes a mutant YFP that does not fluoresce, but can be detected via immunostaining. This feature facilitates screening of Cre-negative Brainbow mice to determine the number and type of cells in which the construct is expressed. The photostability of Brainbow-3.2 is improved due to the addition of a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), commonly used to increase transgene protein levels.

Brainbow Variants and Applications Beyond the Mouse Brain

In addition to improving the Brainbow system, Sanes and Lichtman also developed a complementary Flpbow construct that is functionally similar to Cre-based Brainbow, but is controlled by FLP/FRT recombinase. When

BRAINBOW (CONT'D)

placed under different promoters, Brainbow and Flpbow can be used to label distinct cell populations.

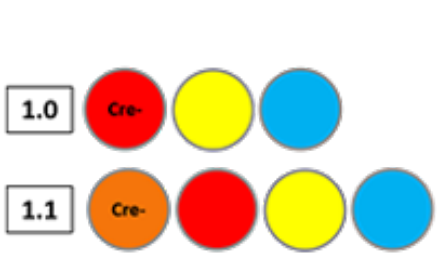
To decrease the animal breeding necessary to produce animals with Brainbow transgenes and Cre, Cai et al. also created an Autobow construct containing both Cre and XFPs. Cre production drives recombination and XFP selection, followed by Cre self-excision. These constructs are stably maintained through at least six generations.

In addition to the neuronal pThy1-Brainbow constructs, Addgene also has two Brainbow adeno-associated viral vectors (AAV) available - AAV-EF1a-BbChT and AAV-EF1a-BbTagBY. These constructs contain two XFPs each due to size limitations associated with AAV; co-infection with both constructs produces a minimum of 8 colors. The use of AAV provides spatial and temporal control without the need for germline modification, and enables Brainbow to be used in a variety of species.

More variants have been created by other labs, including R26R-Confetti described in Hugo J. Snippert, et al. (2010) and the MAGIC Marker strategy described in Karine Loulier, et al. (2014).

Currently, Brainbow techniques are also being applied to study model organisms such as *Drosophila* and zebrafish. Brainbow in *Drosophila* has aided in the mapping of neural circuits, such as connections between motor neurons and the neuromuscular junction. In zebrafish, the method has become very useful in lineage tracing; "Zebrabow" was used to trace the development of the corneal epithelium.

For scientists interested in neuroscience and development, Brainbow is a valuable tool to mark and follow single cells due to the wide array and high stability of colors. Sanes and Lichtman estimate that Brainbow's colorful labeling has decreased the mapping time for a given section of brain by at least an order of magnitude. It is clear that further refinements of the Brainbow technique will provide important insights into the complicated physical organization of the brain and other biological systems.

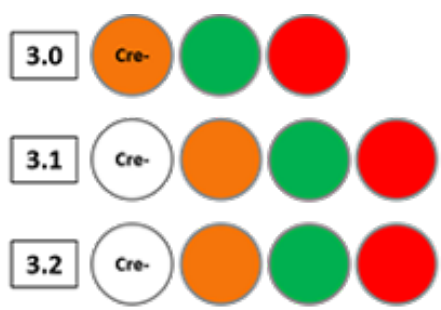


Brainbow-1:
Cre-mediated deletion
- ***1.0***: 3-color (default = red)
- ***1.1***: 4-color (default = orange)

Key points
- Transient or constitutive Cre
- Default fluorescence
- Photoinstability
- No immunostaining

Brainbow-2:
Cre-mediated deletion/inversion
- ***2.1***: 4-color (default = green)

Key points
- Transient Cre only
- Default fluorescence
- Photoinstability
- No immunostaining



Brainbow-3:
Cre-mediated deletion
- ***3.0***: 3-color (default = orange)
- ***3.1***: 3-color, non-fluorescent default
- ***3.2***: 3-color, non-fluorescent default

Key points
- Transient or constitutive Cre
- Photostable fluorophores
- Immunostaining possible
- No default fluorescence (3.1 and 3.2)
- WPRE addition (3.2)

BRAINBOW (CONT'D)

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FLUORESCENT PROTEIN TIMERS

By Tyler Ford | April 12, 2017

Even before fluorescent proteins (FPs) came into wide use, there were a variety of ways to monitor cell, organelle, and protein localization. For instance, you might dye your cells and look at them under a microscope, fractionate samples to isolate particular organelles and their contents, or perform *in situ* hybridization experiments. In many cases fluorescent proteins have usurped old methods or complemented them in ways that make them much easier. A special class of FPs, the FP timers, add an entire new dimension to monitoring localization; using FP timers, researchers can look at a single image of a cell and understand how protein localization changes over time.

Hallmarks of Fluorescent Protein Timers

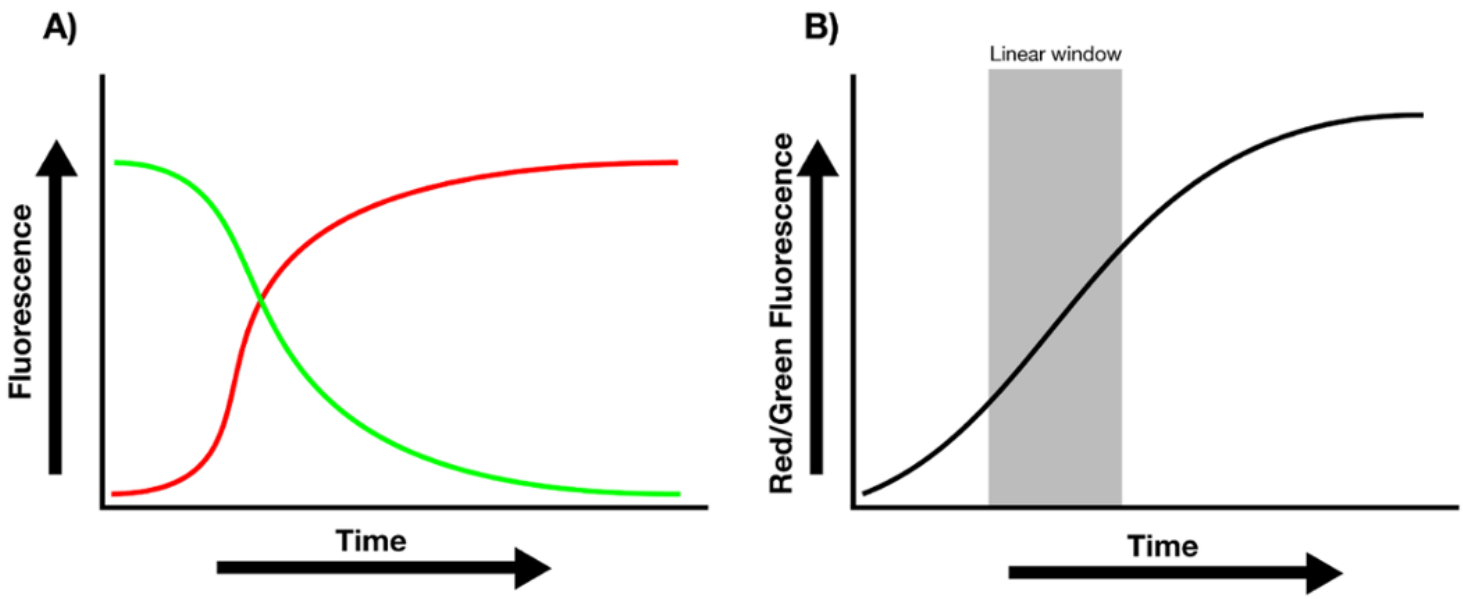

Figure 1: Theoretical properties of an FP timer. A) This theoretical timer starts off fluorescing green. Overtime, the green fluorescence decays and the timer begins fluorescing red. B) The ratio of red to green fluorescence is predictable over time and can be used to determine the amount of time a particular pool of FP timers or FP timer fusions has been expressed. Age is easiest to predict in the linear window.

When FP timers are first expressed, they predominantly fluoresce one color but slowly mature until they predominantly fluoresce a second color. This change is usually hypothesized to be a result of chromophore oxidation. Some of the more popular timers (whether as part of protein fusions or expressed independently) originally fluoresce green or blue and, when they mature, fluoresce red. The ratio of the mature color (red) to the immature color (green or blue) within a population of timer indicates the age of the pool: the higher the ratio, the older the pool.

FP timers have been around since 2000 when Terskikh et al. reported the production of dsRed E5. This timer predictably transitions from green to red fluorescence (500 to 580 nm) over the course of 18 hours in vitro (see figure 1B in Terskikh et al.) and even displays predictable kinetics over 14 hours of expression in *C. elegans* embryos. However, this initial timer was a tetramer with a propensity to aggregate in cells (Tsuboi et al.), limiting its use. Nonetheless, its creators noticed a few properties that have now become hallmarks of ideal FP timers:

1. Ratiometric Determination of Expression Time

The ratio of mature to immature fluorescence from the FP timer is dependent on the total expression time

FLUORESCENT PROTEIN TIMERS (CONT'D)

but independent of protein concentration. Calibration curves correlating this ratio to the total expression time can therefore be used to determine how long a pool of FP timers has been expressed, either in whole cells or as a pool of fusion proteins localized to a specific region of the cell. For example, if the ratio of red/green fluorescence falls within the linear window for the theoretical timer shown in Figure 1B, you can use the equation fit to this linear region to solve for total expression time.

2. Timer Functionality with a Single Gene

Although it is possible to make a timer system composed of two separate FPs (one that matures quickly and the other slowly, see Verkhusha et al.), one benefit of the common FP timers is that you can monitor age by expressing a single FP-timer protein or protein fusion. This one protein method simplifies cloning and makes your experimental setup less cumbersome.

3. Portability Across Multiple Systems

Terskikh et al. showed that their versatile timer could be used *in vitro*, in mammalian cells, in *C. elegans*, and in *Xenopus* embryos. Of course, timers may be sensitive to changes in oxygen, temperature, and pH, but timer activity can be calibrated to these different conditions, enabling the use of a given timer in many experimental settings.

Monomeric Fluorescent Protein Timers

Since the production of the initial FP timer in 2000, researchers have realized how problematic oligomerization-prone tetrameric fluorescent proteins can be. Cytotoxicity, improper localization, and decreased functionality are all possible consequences of FP oligomerization, as Erik Snapp's section shows. To avoid these issues, Subach et al. and Tsuboi et al. developed monomeric FP timers with less propensity to aggregate.

These monomeric FP timers (mK-GO from Tsuboi et al. and the FT series from Subach et al.) were derived from previously developed monomeric fluorescent proteins mKO and mCherry, respectively. While Tsuboi et al developed mK-GO somewhat serendipitously while attempting to enhance mKO in other ways, Subach et al made a concerted effort to develop their FT series using their knowledge of protein structure and saturation mutagenesis. mK-GO matures from green to red, and the FT series matures from blue to red (see Table 1 for emission and absorption spectra). Subach et al's directed mutagenesis also produced the three separate timers shown in the table: Fast-FT, Medium-FT, and Slow-FT. These display progressively longer blue fluorescence maturation times and varied blue-red maturation times (Table 1). These separate proteins should be useful for monitoring cellular events occurring at varied time scales.

Applications of Fluorescent Protein Timers

FP timers have been used to:

- Monitor dynamics of vesicle fusion and release from the plasma membrane by fusing various vesicle cargoes to FP timer mk-GO (Tsuboi et al.)
- Monitor gene expression in the developing pancreas by placing FP timer DsRed-E5 under the control of Neurog3 (a gene controlling pancreatic differentiation) in mouse embryos (Miyatsuka et al.)
- Distinguish between models for trafficking to the lysosome by fusing Medium-FT to LAMP-2A, a lysosome-associated membrane protein, and following its subcellular localization overtime (Subach et al.)

FLUORESCENT PROTEIN TIMERS (CONT'D)

- Monitor protein expression dynamics in a synthetic circuit (Saxena et al.)

Table 1: Monomeric FP Timers and Associated Plasmids from Subach et al.

Protein	Exciation (nm)	Emission (nm)	Brightness	pKa	Matu-ration	Plasmids
Fast-FT	403 (blue), 583 (red)	466 (blue), 606 (red)	14.9 (blue), 6.8 (red)	2.8/4.1	7.1 h (red)	pFast-FT-N1 pTRE-Fast-FT pBAD/HisB-Fast-FT
Medium-FT	401 (blue), 579 (red)	464 (blue), 600 (red)	18.4 (blue), 5.8 (red)	2.7/4.7	3.9 h (red)	pMedium-FT-N1 pTRE-Medium-FT pBAD/HisB-Medi-um-FT
Slow-FT	402 (blue), 583 (red)	465 (blue), 604 (red)	11.7 (blue), 4.2 (red)	2.6/4.6	28 h (red)	pSlow-FT-N1 pTRE-Slow-FT pBAD/HisB-Slow-FT

In a general sense, FP timers can be used in any situation where one wants to understand the relationship between the age of a cell, protein, or cellular structure and a particular biological event (trafficking to a subcellular location, start of gene expression, development of a cell structure, etc). FP timers should therefore find use in studies of animal development where events like the patterning of nascent tissues and the formation of limbs are correlated with changes in gene expression. In a developing embryo, for example, a researcher could determine whether or not newly synthesized or long-lived transcription factors control gross changes in gene expression as new body sections are formed. In synthetic biology, FP timers might be able to tell researchers whether old or new cells are better at producing a compound of interest, thus allowing them to optimize the compound production process.

How have you used FP timers? Are you planning on using an FP timer in a new and creative way? Let us know!

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FLUORESCENT PROTEIN TIMERS (CONT'D)

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LUMINESCENT IMAGING WITH NANO-LANTERNS

By Mary Gearing | August 26, 2015

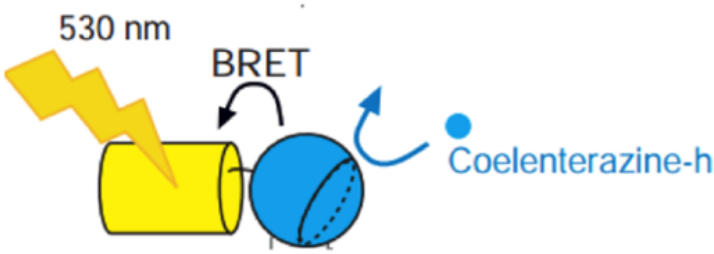

Figure 1: After feeding a substrate to a nano-lantern (coelenterazina-h here), energy is transferred from the luciferase half of the nano-lantern (blue) to the fluorescent protein half (yellow) allowing the fluorescent protein to emit light. Adapted from Suzuki et al 2016.

Fluorescent imaging techniques have become indispensable tools for molecular and cell biologists over the last two decades, but their use can be limited by a few caveats. Since fluorescent proteins (FP) require external light activation, you can't use fluorescence to monitor processes directly affected by light. Long-term light exposure can also lead to cellular phototoxicity, and experimental success can be affected by both autofluorescence and photobleaching. Researchers have long been interested in using luminescence to get around these issues, but this solution wasn't practical due to the low intensity of luminescent proteins. To make luminescent imaging a reality, Addgene depositor Takeharu Nagai and colleagues at Osaka University have developed the Nano-lantern technology. Nano-lanterns contain a Renilla luciferase variant fused to an FP; when supplied with a luciferase substrate, the luciferase transfers energy to the FP, resulting in a fluorescent signal. Since their first publication in 2012, the Nagai laboratory has assembled a collection of multicolored nano-lanterns for use in various applications, including optogenetics, biosensors, and fusion proteins.

Solving the Problems of Low Luminescence

By itself, Renilla luciferase has a low quantum yield, and when transiently expressed in cells, generates much less signal than comparable fluorescent proteins. This weak emission hasn't stopped some scientists from using chemiluminescent proteins for imaging, but it has precluded widespread adoption of the technology.

In 2012, Saito et al. described the first Nano-lantern, a fusion of a mutagenized Renilla luciferase (RLuc8) with Venus. This luminescent technology is based on the principle of bioluminescence resonance energy transfer (BRET). Photons emitted by a Renilla luciferase variant are used to excite the fused fluorescent protein. This technique is very similar to the widely used FRET, but it eliminates the need for an excitation light source. Instead, a luciferase substrate like coelenterazine is supplied in the culture medium to allow RLuc8 to produce photons.

The original nano-lantern emitted yellow-green light and exhibited 3-5 fold increased brightness over previous BRET probes, with sufficient signal for *in vivo* and *in vitro* imaging. Saito et al. used this Nano-lantern to image labeled tumors in freely moving mice injected intravenously with coelenterazine, with increased sensitivity and faster imaging compared to previous publications. They also developed calcium, cAMP, and ATP sensors based on Nano-lanterns, showing the technique's versatility and adaptability to many different contexts.

Improving Nano-Lanterns

In 2015, Takai et al. expanded the Nano-lantern color palette to include cyan and orange Nano-lanterns and showed that these three luminescent proteins can be used together in cells. As Nano-lanterns are not subject to photobleaching/phototoxicity, they successfully imaged multiple subcellular compartments over several minutes using Nano-lantern fusion proteins. To further improve long-term imaging, which is limited by depletion of luminescent substrate, they synthesized diacetyl coelenterazine-h. Unlike coelenterazine-h, diacetyl coelenterazine-h does not autooxidize and produce background fluorescence, so higher concentrations of the compound can be added to the media. Cellular esterase then converts diacetyl coelenterazine-h to

LUMINESCENT IMAGING WITH NANO-LATERNS (CONT'D)

coelenterazine-h, producing a constant supply of substrate. Using this compound to excite the Nano-lanterns, Takai et al. continuously imaged cells for about 4 hours!

The latest enhanced Nano-lanterns (eNL) described in Suzuki et al. improve the brightness of previous Nano-lanterns by 2-4 fold and permit five-color fluorescent imaging! These eNL constructs use the brightest characterized luciferase, NanoLuc, and its substrate furimazine. In addition to the previously described applications, the enhanced signal of eNL allows their use for the first time in single molecule imaging.

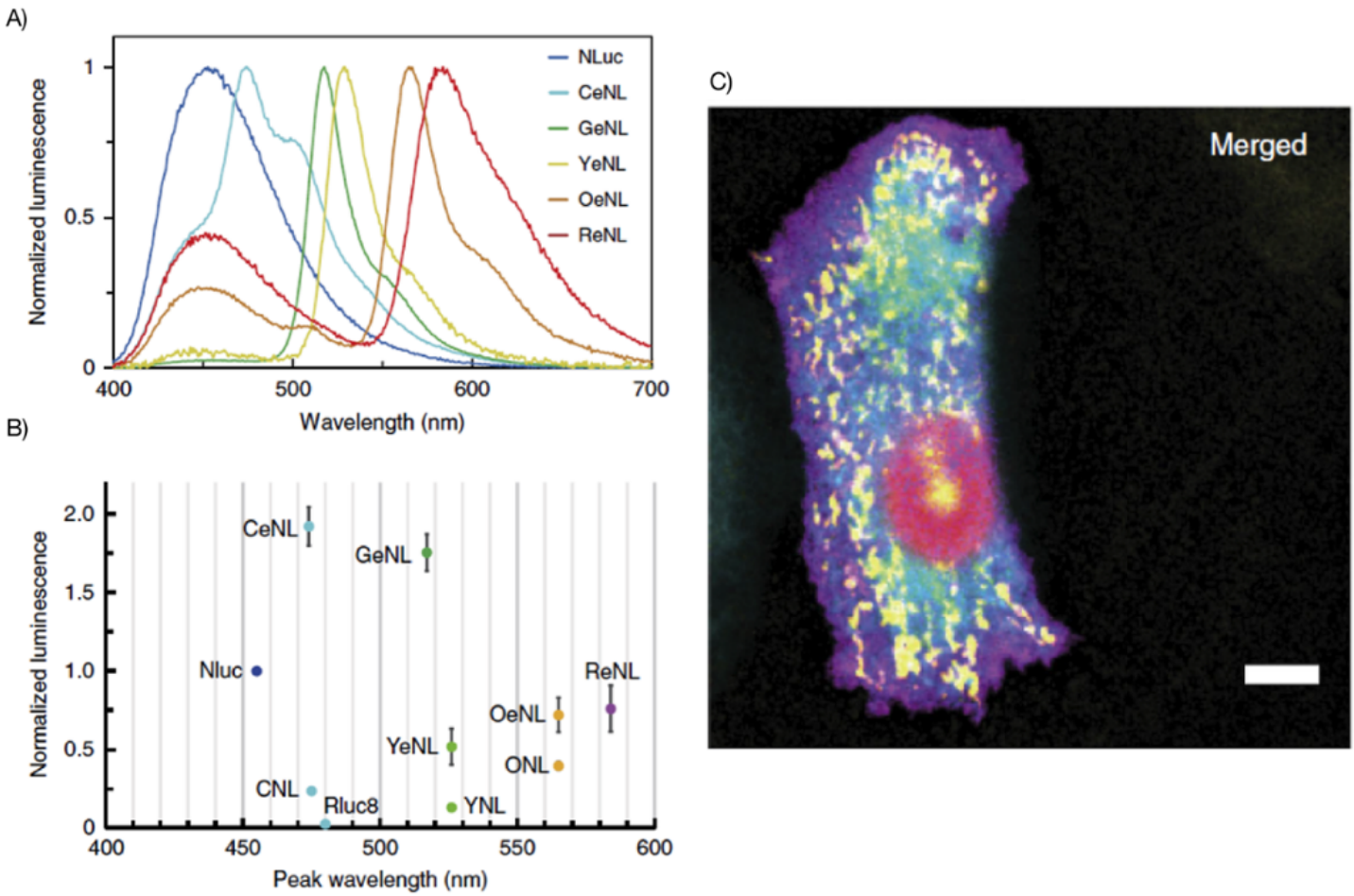

Figure 2: Nanolanterns come in a variety of colors (A,B) and can be used for multicolor imaging (C). Adapted from Suzuki et al 2016.

Nano-Lanterns and Your Experiments

The Nagai laboratory has a done a wonderful job characterizing Nano-lanterns and eNL for many different applications. For long-term imaging, especially where photobleaching and phototoxicity are a concern, these constructs come in very handy. This approach is especially suited to complement optogenetic tools, where light used for excitation can cause unintended activation of the optogenetic system. Biosensors based on Nano-lantern technology are also very robust, with an eNL-based calcium sensor displaying a 500% signal change upon calcium binding. Suzuki et al. also envision eNLs as good reporter genes for endogenous protein fusions created using CRISPR/Cas9. eNLs offer high luminescent signal for imaging of low-copy number proteins, whereas such detection is difficult using fluorescent imaging due to autofluorescence and a high signal-to-noise ratio.

LUMINESCENT IMAGING WITH NANO-LATERNS (CONT'D)

Despite the many benefits of Nano-lantern technology, there are a few drawbacks associated with luminescent imaging. First, a luminescent substrate is required for imaging, and it is important to verify that this substrate does not alter cellular physiology in your system. Second, optical sectioning cannot be conducted with luminescence imaging since there is no external light source. Therefore, especially thick samples may not be appropriate for use with Nano-lantern technology. Third, multicolor imaging using Nano-lanterns requires a linear unmixing algorithm to separate the various colors. In fluorescent imaging, you use the excitation wavelength to specify which FP emission you'll capture. Since the Nano-lantern system does not use external light for excitation, you instead have to rely on differences in emission wavelength. Filtering the emission wavelength partially separates the signals of various Nano-lanterns, but it does not isolate each Nano-lantern, so Takai et al. used a linear unmixing algorithm to decode the individual colors. The development of this algorithm is explained further in Takai et al.

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PHOTOACTIVATABLE FLUORESCENT PROTEINS

By Michelle Cronin | April 19, 2017

Fluorescent proteins (FPs) offer scientists a simple yet powerful way to tag cellular proteins and investigate protein localization, interaction, and expression. However, one caveat of FP-protein fusions (FP-chimeras) is that they undergo normal protein turnover. FP-chimeras are continuously synthesized and degraded within the cell, so at any given time, an FP-chimeric protein may be at any one of many stages of synthesis and degradation. For this reason it is virtually impossible to determine specific protein turnover or temporal expression using standard FP-chimeric proteins.

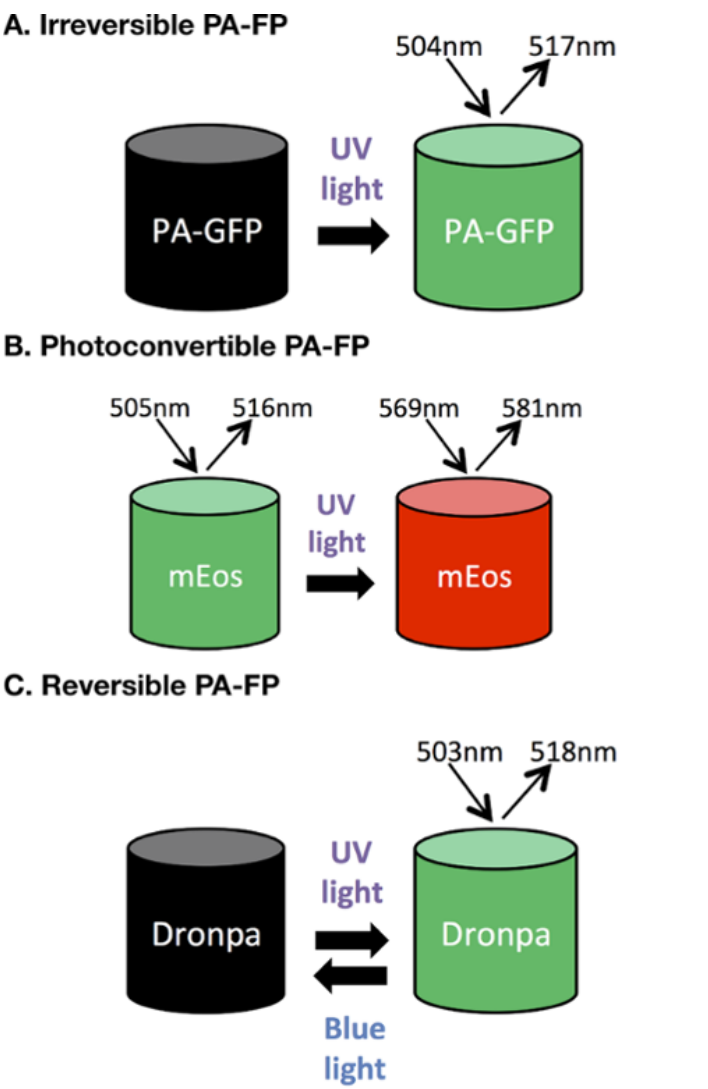

Figure 1. Examples of PA-FPs. A) PA-GFP is an irreversible PA-FP. Upon UV-illumination, PA-GFP is photoconverted from a dark state to a bright green fluorescent state with excitation/emission of 504/517 nm. B) mEos is a photoconvertible PA-FP (PC-FP). Following UV light, mEos photoconverts from green to red fluorescence with excitation/emission of 569/581 nm. C) Dronpa is a reversible PA-FP. UV light photoconverts Dronpa from a dark state to a green fluorescent state with excitation/emission of 503-518 nm. Blue light quenches Dronpa back to the dark state. UV light allows Dronpa to photoconvert back to a green state (Figure adapted from Wang et al. 2008).

Photoactivatable fluorescent proteins (PA-FPs) are fluorescent proteins that display unique changes in their spectral properties upon exposure to a specific wavelength of light. PA-FPs can be activated from low fluorescent states to high fluorescence states, they can change from one fluorescent color to another, or their fluorescence can be switched on and off reversibly. This ability to modulate fluorescence allows scientists to observe single fluorescent molecules that would otherwise be missed with conventional FP imaging. PA-FPs are divided into 2 main categories - those with irreversible and reversible photoactivation - and have enabled sophisticated imaging techniques.

Irreversible Photoactivation

Irreversible PA-FPs can be switched on from a state of dim or no fluorescence, to a brighter fluorescent state by exposure to a specific wavelength of light. The switch or photoconversion usually takes less than a second. The first successful irreversible PA-FP reported was PA-GFP. PA-GFP was derived from Jellyfish (*Aequorea victoria*) GFP (wtGFP) by mutating Threonine 203 to Histidine (T203H) (1). wtGFP normally contains a mixed population of neutral (protonated) and anionic (deprotonated) forms of the chromophore which contribute to the 2 peaks of wtGFP's excitation spectrum- a major 397 nm peak and minor 475 nm peak, respectively. When wtGFP is illuminated with UV-violet light (~400 nm), the chromophore photoconverts predominantly to the anionic form, causing increased fluorescence when excited with 488nm blue light. The T203H mutation in PA-GFP generates a chromophore population that is more neutral, causing PA-GFP to display almost no fluorescence when illuminated with 488 nm light. However, when UV-violet light is presented first, it causes irreversible photoconversion of the chromophore from the neutral to the anionic form, which allows PA-GFP to exhibit up to a 100-fold increase in fluorescence when illuminated with blue

PHOTOACTIVATABLE FLUORESCENT PROTEINS (CONT'D)

light (1).

Similar to PA-GFP, scientists later developed red fluorescent PA-FPs by performing several rounds of random mutagenesis screening for enhanced RFP variants. PA-mCherry (E26V/A58T/K69N/L84F/N99K/S148L/I165V/Q167P/L169V/I203R) (2) and PA-mRFP1(S146H/I161V/I197H) (3) were derived from DsRed and mRFP, respectively. When illuminated with violet light, both variants are photoconverted to forms that emit brighter red fluorescence. Since emission of green light is more phototoxic to cells, most scientists working on *in vivo* imaging models perfer the PA-RFPs over PA-GFP.

Another class of irreversible PA-FPs are the photoconvertible fluorescent proteins (PC-FPs). PC-FPs can convert from one fluorescent color to another (e.g. green to red, or cyan to green).

For example, mEosFP (named after the goddess of dawn in Greek mythology) switches from green to red fluorescence. mEosFP exposure to UV light causes an irreversible cleavage near the chromophore, resulting in emission of red fluorescence (4). The dimeric or tandem dimer versions of EosFP are preferred over the monomeric one since the formation of mEosFP's chromophore requires a temperature below 30 °C and this is not ideal for experiments in mammalian cells.

Dendra2 is another monomeric PA-FP which unlike mEosFP, can form it's chromophore at a more comfortable 37 °C (5). Dendra2 photoconverts from green to red fluorescence when exposed to blue light, which is less phototoxic to live tissue than the UV light used by other PA-FPs. Several other irreversible PA-FP spectral variants have been discovered such as Kaede and PS-CFP2. (see Table 1).

Table 1: Properties of Selected Irreversible Photoactivatable Fluorescent Proteins

Protein	Oligomeric State	Activating Light	Pre/ Post Color	Contrast (Fold Change in Fluorescence)	Brightness	Excitation/ Emission (nm)
PA-GFP	Monomer	UV-Violet	Dark/ Green	~200	Medium	504/ 517
PS-CFP2	Monomer	UV-Violet	Cyan/ Green	>2,000	Medium	490/ 511
Kaede	Tetramer	UV-Violet	Green/ Red	~2,000	High	572/580
tdEos	Tandem	UV-Violet	Green/ Red	N/A	High	569/ 581
mEos	Monomer	UV-Violet	Green/ Red	N/A	High	573/ 584
KikGr	Tetramer	UV-Violet	Green/ Red	>2,000	High	583/ 593
mKikGr	Monomer	UV-Violet	Green/ Red	560	High	580/ 591
Dendra2	Monomer	UV-Violet or Blue	Green/ Red	4,500	High	553/ 573
PA-mCherry	Monomer	UV-Violet	Dark/ Red	>3,000	Medium	570/ 596

Reversible Photoactivation

In contrast to irreversible PA-FPs, reversible PA-FPs (also known as photoswitchers) can be photoswitched

PHOTOACTIVATABLE FLUORESCENT PROTEINS (CONT'D)

from a dark state to a bright fluorescent state ("kindling"), and from a bright state to a dark non-fluorescent state ("quenching"). The photoswitch can occur multiple times by activating with two distinct wavelengths of light (see Table 2).

The best known reversible PA-FP is the naturally occurring Dronpa protein (named after dron, a ninja term for vanishing, and pa, for photoactivation) (6). Dronpa is photoactivated by blue wavelengths of light, causing emission of green fluorescence, but then further exposure to blue light also inactivates the chromophore, leading to a non-fluorescent state. Dronpa can be reversibly reactivated by exposure to UV light. The fact that blue light both activates and inactivates Dronpa is not ideal, since this means Dronpa is quickly inactivated and less photons are emitted leading to dimmer fluorescence. In fact, many of the early reversible PA-FPs also display this "negative" photoswitching.

In 2008, scientists used mutagenesis screens to find a version of Dronpa that doesn't become inactivated as quickly. Padron is a monomeric, reversible PA-FP derived from mutating Dronpa (T59M/V60A/N94I/P141L/G155S/V157G/M159Y/F190S) (7). Padron's "positive" switching behavior is the opposite of Dronpa; when exposed to blue light, Padron is photoconverted from a dark state, to an activated state, emitting bright green fluorescence. When exposed to UV light Padron is inactivated.

The rs-Cherries (8) are another group of reversible PA-FPs. These were engineered to be the first reversible, monomeric red fluorescent PA-FPs. rsCherries have a high background fluorescence, but their single molecule brightness and ability to switch from a dark state to a red fluorescent state make them useful for 2-color imaging with green PA-FPs.

Table 2: Properties of Selected Reversible Photoactivatable Fluorescent Proteins

Protein	Oligomeric State	Activating Light	Pre/ Post Color	Contrast (Fold Change in Fluorescence)	Brightness	Excitation/ Emission (nm)
Dronpa	Monomer	UV-Violet	Dark/ Green	N/A (High)	High	503/ 518
Padron	Monomer	Blue	Dark/ Green	N/A (High)	Low	503/ 518
rsCherry	Monomer	Yellow	Dark/ Red	7	Low	572/ 610
rsCherryrev	Monomer	Blue	Dark/ Red	20	Low	572/ 610
FP595	Tetramer	Green	Dark/ Red	70 - 1000	Medium	590/ 600

Choosing a PA-FP for Your Experiment

Similar to performing experiments with standard FPs, before choosing a PA-FP for your experiment, there are several parameters to consider, such as brightness, photostability, pH stability, chromophore maturation rate at 37 °C, turnover rate, and the level of background fluorescence. For any PA-FP application, the brighter the fluorescence the better. More fluorescence usually means more photons are being emitted, making it easier to capture a clear image above background.

The majority of PA-FPs come in 2 flavours- monomeric and tetrameric. Tetrameric PA-FPS are better for whole cell imaging and studying organelle trafficking. Monomeric PA-FPs are usually preferred for studying single

PHOTOACTIVATABLE FLUORESCENT PROTEINS (CONT'D)

protein characteristics, as the monomeric state is less likely to interfere with endogenous protein structure and function. By generating monomeric PA-FPs, there is less chance that the chimeric protein will oligomerize, disrupting cellular function and localization, or leading to protein aggregation.

Another important parameter to consider is the contrast ratio- a comparison of PA-FP brightness before and after photoactivation. A high contrast ratio usually indicates that a PA-FP has a low level of spontaneous fluorescence in the absence of photoactivation resulting in an increased signal to noise ratio. PA-FPs with higher contrast ratios may give you experimental results that are easier to interpret.

The light intensity used to activate the PA-FP should also be considered. If the light intensity needed for activation is too high, the photoactivation step can actually harm your cells before your experiment even begins! If you are performing multicolor experiments, you will want to be sure that the light used to activate the first PA-FP does not photobleach the second, and vice versa. Finally, as every microscope will have it's own quirks (level of zoom, intensity, possible excitation lasers) it is also important to optimize your activation protocol for your particular set up.

Applications of PA-FPs

Some of the most important applications for PA-FPs involve enhancing optical live imaging of proteins, organelles, and cells. Similar to FPs, PA-FPs allow for non-invasive labelling, but in contrast, PA-FPs offer a reduced potential for photobleaching and phototoxicity that standard FPs might induce though their continuous excitation. By generating PA-FPs fused to intracellular proteins, scientists can track single protein localization, turnover, and trafficking, as well as organelle trafficking and dynamics (fission, fusion, etc). For example, by engineering PA-GFP tagged to a mitochondrial matrix protein (mito-PAGFP) the Youle Lab was able to visualize and quantify the fusion dynamics of mitochondria in healthy and apoptotic cells (9).

PA-FPs have helped to revolutionize the field of super resolution microscopy. PA-FPs, especially the monomeric, reversible type, are very well suited for super resolution imaging, where the power of the technique relies heavily on the same fluorophore being imaged multiple times in order to reconstruct an image. The best PA-FPs used for super-resolution imaging usually have the following characteristics-high contrast ratios, bright fluorescence, low spontaneous activation, and photoswitching between two fluorescence wavelengths.

Indeed, within the world of super resolution microscopy, PA-FPs have helped create several new imaging methods, including photoactivation-localization microscopy (PALM, also FPALM) (11). For PALM, a small area containing inactive PA-FPs is photoactivated, imaged, and then photobleached. Additional PA-FP molecules are then activated in the same manner, until enough molecules have been analyzed to allow for construction of an image, providing detailed protein localization information in cells. A point to keep in mind- although PA-FPs are vastly improved over the conventional FPs for super-resolution imaging, they still do not emit the large number of photons achievable with photoswitchable dyes.

Correlative light and electron microscopy (CLEM) is an imaging technique that combines electron microscopy (EM) with fluorescence localization data. However, this technique requires harsh fixation conditions to preserve cellular structures (0.5-1% osmium tetroxide). These conditions destroy most PA-FPs, but, in 2015, the Looger Lab designed 2 variants of EosFP that can withstand these conditions- mEos4a and mEos4b (12). Both variants are more monomeric than mEos2 (mEos4b is completely monomeric) and display bright and photostable green and red states.

PHOTOACTIVATABLE FLUORESCENT PROTEINS (CONT'D)

Beyond these imaging applications PA-FPs have been developed for use in yeast, as parts of biosenors, and much more. Do you have a favorite use for PA-FPs? Let us know in the comments section below!

Currently, there are over 20 different varieties of PA-FPs available to the scientific community, and new variants are still being engineered. By combining PA-FPS with superresolution microscopy, scientists now have at their fingertips the tools to probe cellular dynamics and function with extraordinary molecular detail.

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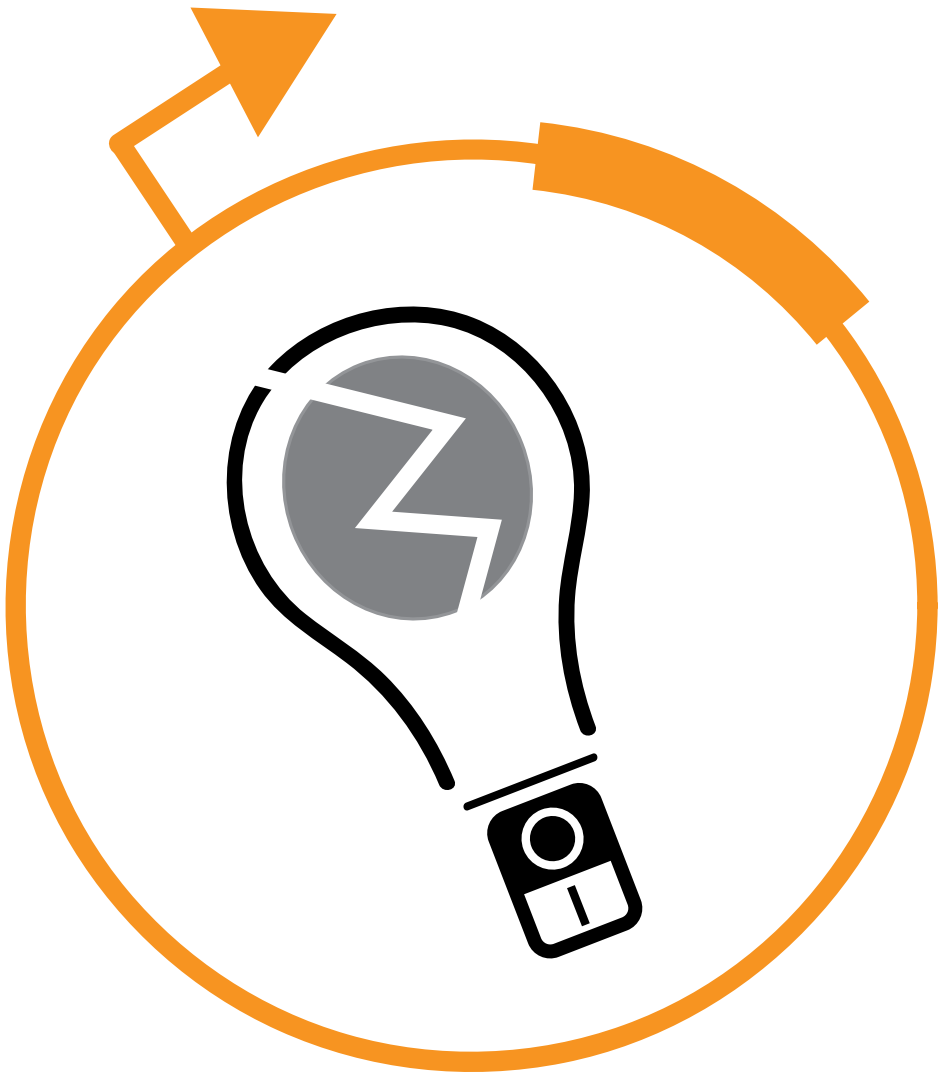
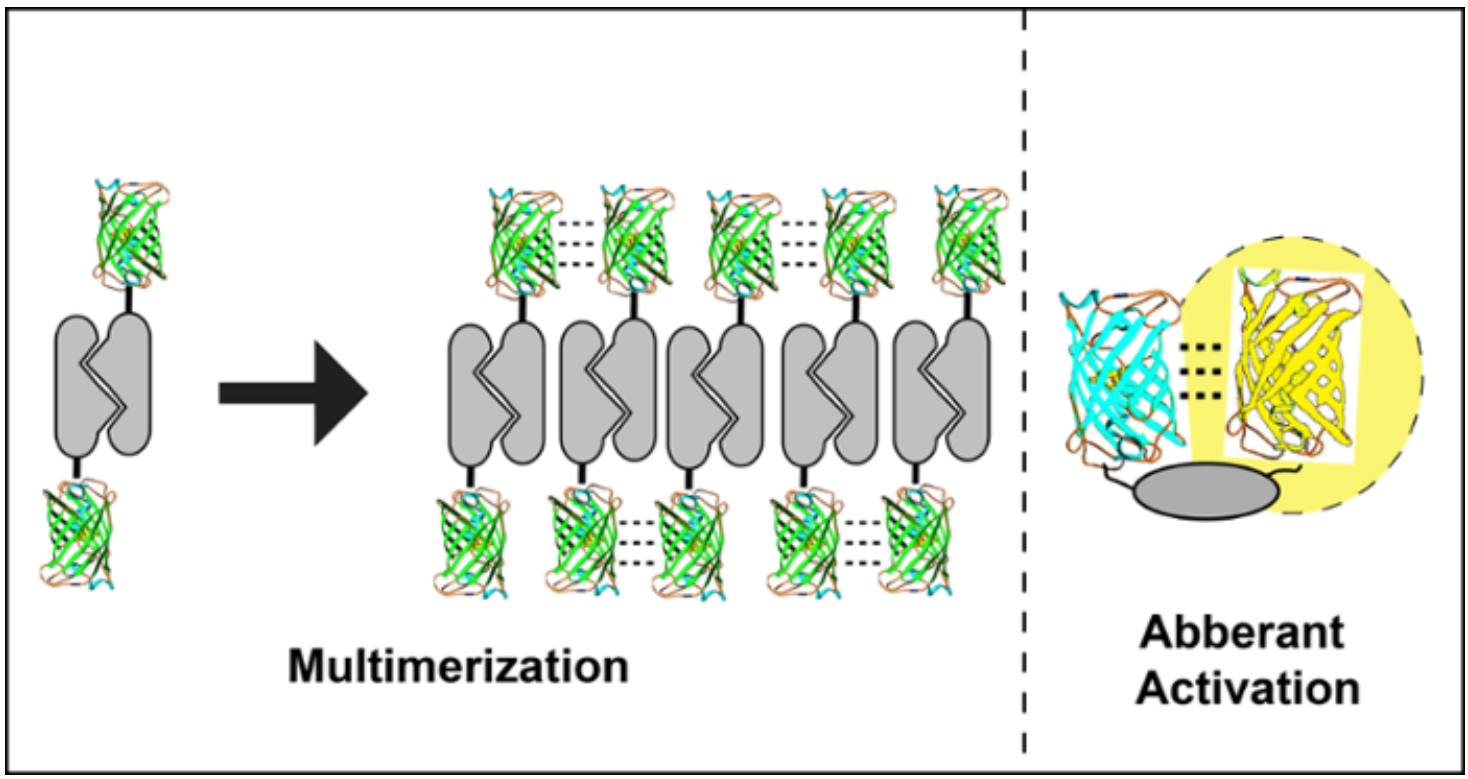
CHAPTER 9: FLUORESCENT PROTEIN PITFALLS



FLUORESCENT PROTEIN OLIGOMERIZATION

By Erik L. Snapp | April 19, 2015



Stop using EGFP/GFP for fusion proteins! Despite multiple studies in high profile journal articles, many researchers remain unaware that EGFP/GFP is prone to forming noncovalent dimers. This property of EGFP can lead to significant artifacts.

If you're using green fluorescent protein or Enhanced Green Fluorescent Protein (GFP/EGFP) for a transcriptional reporter or as a general cytoplasmic label of cells, there's no problem. You're OK. However, if you fuse your protein of interest (POI) to GFP to study the protein's behavior in cells, in solution or something in between, you are using a tag with a serious drawback. The standard EGFP plasmid that used to be sold by Clontech and is in a freezer box in just about every lab in the world, is not inert. In all seriousness, EGFP/GFP has a real nontrivial propensity to noncovalently dimerize. That means that your POI fused to GFP or another fluorescent protein (FP) could be forming dimers in cells. Why should you care? Three simple ways a dimeric FP could ruin your day (and experiment) are listed below. Solutions to avoid these all too common issues follow.

It's a Matter of Concentration

Most FPs in nature are prone to dimerization (i.e. EGFP) [1, 2] or even form obligate tetramers (i.e. DsRed) [3]. This is a problem for fusion proteins. One of the major applications of FPs is to visualize the localization, dynamics, and behavior of a POI. As an investigator, you want a fusion tag to be inert, not to produce artifacts in your experiments. Considerable effort has gone into making FPs monomeric, yet many investigators remain ignorant of FP dimerization. Equally problematic, several reportedly monomeric FPs are not actually monomeric, at least in practical terms. The propensity of a particular type of molecule to form a dimer depends on its molecular affinity, termed the dissociation constant or Kd, and its concentration. The smaller the Kd, the more likely molecules of a particular type will interact. A protein with a nanomolar Kd will exist primarily as a

FLUORESCENT PROTEIN OLIGOMERIZATION (CONT'D)

dimer in cells while a protein with a high micromolar or low millimolar Kd is unlikely to form dimers in a cell. The Kd of EGFP is 0.11 mM [2]. Following the simple logic outlined above, you might think EGFP is unlikely to form dimers in a cell. Unfortunately, things are not quite so simple.

Concentration is the number of molecules within a volume. In many biological research situations, the relevant volume is that of the cell. For the physical behaviors associated with a molecule's concentration to be accurately predicted, it is assumed that the molecules are homogenously distributed throughout the volume and can freely tumble in 720°. However, if the molecules are confined to a subregion of the cell and/or they cannot tumble in 720°, i.e. they are on a membrane or in an organelle, then their effective concentration will be much higher than if they are homogenously distributed and mobile throughout the cell. If two copies of a molecule are part of a single fusion protein, as in a FRET biosensor, then the local concentration of FPs around that fusion protein is very high. Real world examples of these situations in cells are described below (See Figure 1).

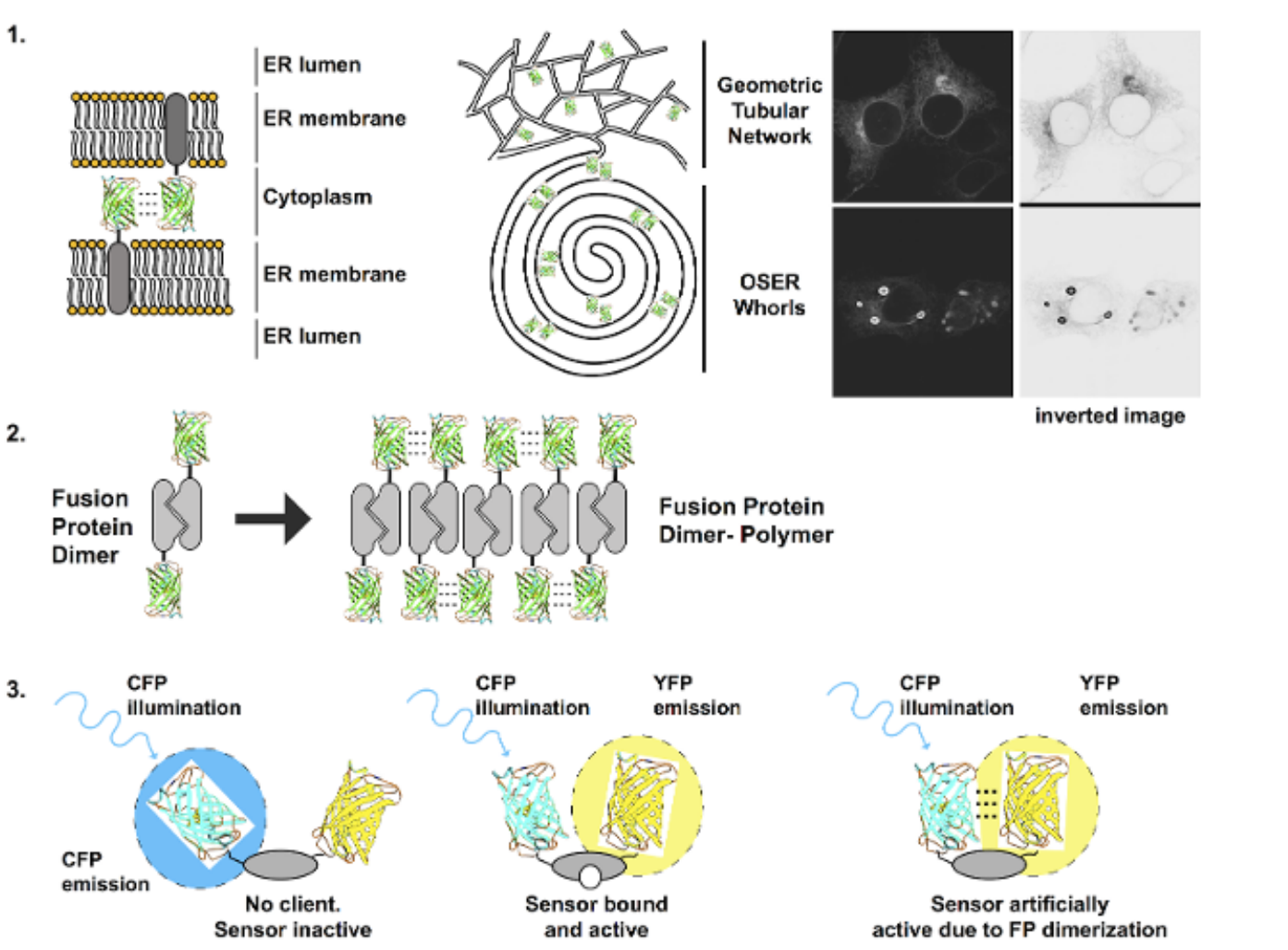


Figure 2. Each problem with dimerizing fluorescent proteins is illustrated. 1. Membrane proteins fused to FPs that dimerize can form dynamic contacts that bring apposing membranes together and can warp the membranes into stacked structures including organized smooth endoplasmic reticulum (OSER) whorls. 2. A dimerizing protein of interest fused to a dimerizing FP has potential to form large stable polymers of dimers. 3. FRET biosensors that contain two dimerizing FPs can exist in an open inactive state, a closed active state, and an artificially active state due to closure by FP dimerization.

FLUORESCENT PROTEIN OLIGOMERIZATION (CONT'D)

1. Transmembrane Fluorescent Protein Fusions

Transmembrane proteins, i.e. receptors and transporters, are integrated into lipid bilayers. Fusion of an FP to a transmembrane protein significantly increases the effective concentration of the FP. The FP is confined to a plane and generally only rotates in 360°, increasing the probability that two FPs will collide with each other. The consequences can be dramatic. For a membrane POI localized to the endoplasmic reticulum, the normally spiderweb-like pattern of tubules can be grossly distorted into dense stacked membranes termed Organized Smooth Endoplasmic Reticulum or OSER if a dimerizing FP is fused to the POI. These structures are large (microns), bright, non-physiological, and difficult to ignore [1, 2].

2. Fusion to an Obligate Dimer or Oligomer

Several cell proteins normally self-associate into homodimers or even higher order oligomers. Unfortunately, FP dimerization coupled with POI dimerization can lead to polymerization of the fusion protein. A dimeric fusion protein will have two FP binding domains forming a building block sufficient to seed polymer formation as fusion protein concentration increases. The polymerized fusion proteins that form as a result will often localize incorrectly and may function improperly.

3. Incorporation into a FRET Biosensor

A FRET biosensor is a reporter that undergoes a conformational change to bring two FPs on a single protein closer together or further apart. Changes in distance alter the ability of one FP to nonradiatively transfer energy to the second FP, often decreasing the fluorescent signal from the first FP and increasing the signal from the second FP. Several early FRET biosensors were made with CFP and YFP, cyan and yellow variants of EGFP equally capable of dimerizing as EGFP. As a result, CFP and YFP dimers can form in the absence of environmental changes that the biosensor is designed to detect resulting in false positives [2, 4].

Solutions to the Polymer Problem

There are some simple solutions available for researchers to deal with all of these problems. The simplest and best solution is to use truly monomeric FPs. EGFP and other GFP family members (superfolder, Emerald, CFP, YFP, BFP, Cerulean, Turquoise, Venus, and Citrine) can be monomerized with an A206K mutation [2]. Addgene has many of these constructs and they are designated mGFP, mVenus, etc. A word of caution is needed because FPs not derived from GFP, i.e. the red FPs, are not necessarily monomeric, even if they were described as monomeric in a publication. The gold standard in assays used to determine if a protein is monomeric is sedimentation equilibrium analytical ultracentrifugation [2]. Some affinity assays used in the papers describing the FPs above including molecular sizing columns and native gels fail to detect EGFP dimerization. Importantly, all of these assays are performed *in vitro*. Assays to detect FP dimerization in *in vivo* cellular contexts, such as the CytERM whorl formation assay, are practical real world measures of dimerization potential that will determine if an FP will dimerize in an experimentally relevant circumstance. For a more detailed discussion see [5].

Problems 1 and 2 depend on protein concentrations. An imperfect solution is to restrict imaging analysis to cells expressing the lowest levels of the fusion protein. This may alleviate artifacts for moderately dimerizing FPs such as EGFP, but not for obligate oligomers such as DsRed or high affinity FPs such as TagRFP. Problem 3 can be solved, in most cases, by using monomeric FPs or FPs from different families, which cannot form

FLUORESCENT PROTEIN OLIGOMERIZATION (CONT'D)

heterodimers (i.e. EGFP and mCherry). These simple strategies can help you make better fluorescent fusion protein reporters for your studies and greatly lower your chances of finding artifactual results.

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FLUORESCENT PROTEIN MISFOLDING

By Erik L. Snapp and Lindsey M. Costantini | October 27, 2015

"You underestimate the power of the Dark Side."

--Darth Vader in "Return of the Jedi"

While Vader was referring to the evil side of a mystical "Force," this quote is equally applicable to many microscopy experiments with fluorescent proteins (FPs) localized to compartments other than the cytoplasm. That is because, unfortunately, some investigators realize too late that they have missed the impact of dark, non-fluorescent, and misfolded FP-fusions on quantitative imaging experiments and cell physiology in general.

Pitfalls of Fluorescent Protein Fusions

Observing a bright signal for the first time after cloning an FP fusion with your favorite protein of interest is exciting and rewarding. But, what if an FP fusion with a resident endoplasmic reticulum (ER) protein instead localizes to the cytoplasm? Or more troubling, what if a significant fraction of the fusion protein molecules fails to correctly fold? Misfolded FPs do not fluoresce. A dark population is not readily apparent and can confound quantitative imaging experiments or even negatively impact cells (see Figure 1).

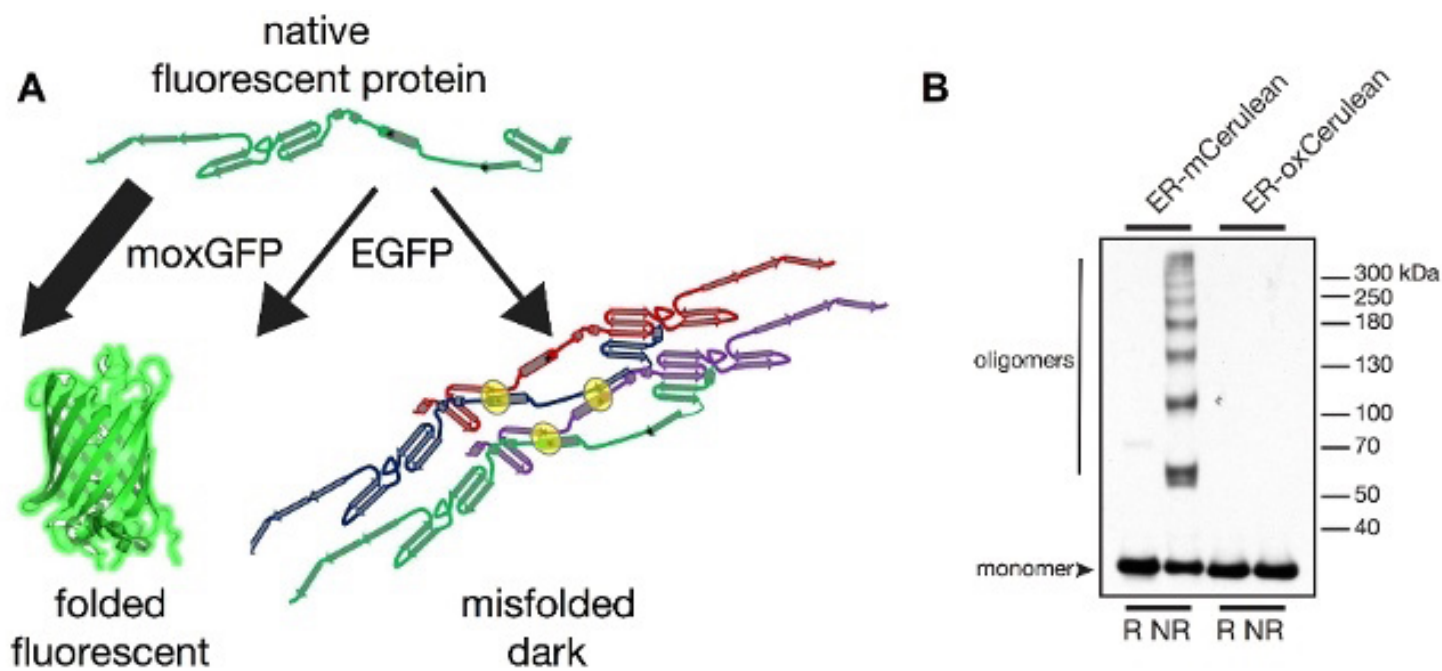

Figure 1. A) In the secretory pathway and other oxidizing environments, standard EGFP is highly prone to forming disruptive interchain disulfide bonds that result in dark FPs. In contrast, moxGFP and other moxFPs lack cysteines and are immune to disulfide bond formation resulting in high efficiency folding of the FP and consequently a robust fluorescent signal. B) Non-reducing gels reveal that a pool of interchain disulfide bonds form between GFP-derived, ER-mCerulean molecules when localized to the ER. These misfolded proteins cannot achieve beta barrel structures and thus are dark. In contrast, the monomerized, cysteineless moxCerulean cannot form misfolded oligomers in the secretory pathway.

When behaving properly, FPs enable researchers to investigate the localization and dynamics of fluorescent protein fusions in live cells in real time. Previously, we have described a number of practical considerations that should be taken into account when deciding where to place an FP within a fusion construct [1,2], i.e. positioning of the FP sequence relative to cell compartment targeting sequences, which often have absolute position requirements. For example, a KDEL ER retrieval sequence only functions at the extreme C-terminus

FLUORESCENT PROTEIN MISFOLDING (CONT'D)

[3]. You must therefore determine your cloning strategy and decide whether you will create an N- or C-terminal fusion based on your experimental needs.

Equally important, before cloning you must consider whether and how well your FP will function in the environment where it will localize [4]. Most investigators are surprised to learn that FPs are often poorly suited for cellular compartments other than the cytoplasm. Generally, FPs evolved in or were engineered for use in cytoplasmic environments. However, approximately 40% of human (and most eukaryotic) proteins localize to chemically distinct subcellular environments, including the organelles that compose the secretory pathway, endocytic vesicles, mitochondria, lysosomes or they are secreted into the extracellular milieu. Many of the resident proteins within these compartments undergo significant post-translational modifications including glycosylation, disulfide bond formation, and proteolytic cleavage. FPs localized to organelles are equally susceptible to these non-native modifications, which can all potentially affect function [5]. We and others have reported that FP cysteine residues form inappropriate disulfide bonds in the secretory pathway (Figure 1) [4,6–8]. Additionally, FPs that encode N-glycosylation consensus sequences are modified with the addition of N-glycans [9]. Both of these posttranslational modifications can disrupt FP folding rendering the protein non-fluorescent.

Unfortunately, accumulated misfolded and nonfunctional (dark) fluorescent proteins cannot be ignored. They have the potential to interfere with the function of resident ER chaperones and may impact cellular function or possibly viability. FP misfolding can even disrupt fusion protein localization. For example, the formation of inappropriate inter-chain disulfide bonds between FPs blocks Golgi complex-localized fusions from properly exiting the ER and fluorescing (see Figure 2) [5]. When using FPs containing two cysteine residues and a sequence intended to localize them to the secretory pathway, a dark pool that is mislocalized to the ER can be detected with immunofluorescence. This population of dark fusions is likely misfolded, blocked from proceeding through the secretory pathway, and retained in the ER.

FPs have been undeniably powerful and successful tools that have enabled numerous cell biology assays. However, there has been a pervasive view that most FPs in a cell will fold and that the fluorescence pattern is equivalent to the distribution of FP-fusion proteins. Our studies and those of others (5, 6, 7, 10, 11) highlight the very real consequences of misfolded dark FP fusions including erroneous quantitation of fusion protein levels. It remains unclear whether the FP misfolding impacts fusion protein function and this could lead to under and overestimation of fusion protein activity. We encourage investigators to characterize FP fusion proteins functionally relative to the untagged protein of interest. To avoid the accumulation of dark fluorescent proteins in your experiments, please consider the following.

How can one ensure that an FP fusion with a non-cytosolic protein functions properly?

We suggest starting by using the newly published oxidative optimized palette of FPs (moxFPs) [5]. The moxFPs overcome the inherent problems of popular fluorescent proteins and were re-engineered by mutating cysteine residues and N-glycosylation consensus sequences in popular FPs. This generated a palette of inert green, cyan, yellow, and blue variants that can be used in various combinations. For example, with standard fluorescent microscope filter sets, users can label multiple proteins of interest with a combination of green and blue or cyan and yellow moxFPs. The inherent brightness and spectral characteristics of the moxFPs are comparable to the non-optimized parental proteins. Our recent publication illustrated the quantitative increase in fluorescent signal achieved when utilizing an optimized moxFP as opposed to standard FPs. The increase in

FLUORESCENT PROTEIN MISFOLDING (CONT'D)

signal is not due to a brighter FP, but rather because of the correct folding and fluorophore formation by most, if not all, of the FP fusions.

Our moxFPs currently represent one of the most inert solutions for imaging in a variety of cellular compartments including the secretory pathway (Figure 2), the inter membrane space of mitochondria and chloroplasts, the extracellular milieu, and gram negative bacteria periplasm. Efforts to tag endogenous genes for proteins in these compartments using CRISPR systems should strongly consider using moxFPs over standard FPs for the reasons listed above. In addition, because the moxFPs are highly monomeric, they are an excellent choice for fusions with integral membrane proteins and membrane associated proteins (i.e. GPI-anchored proteins), which are more susceptible to the oligomerizing effects of many standard FPs. The moxFPs are now available through Addgene.

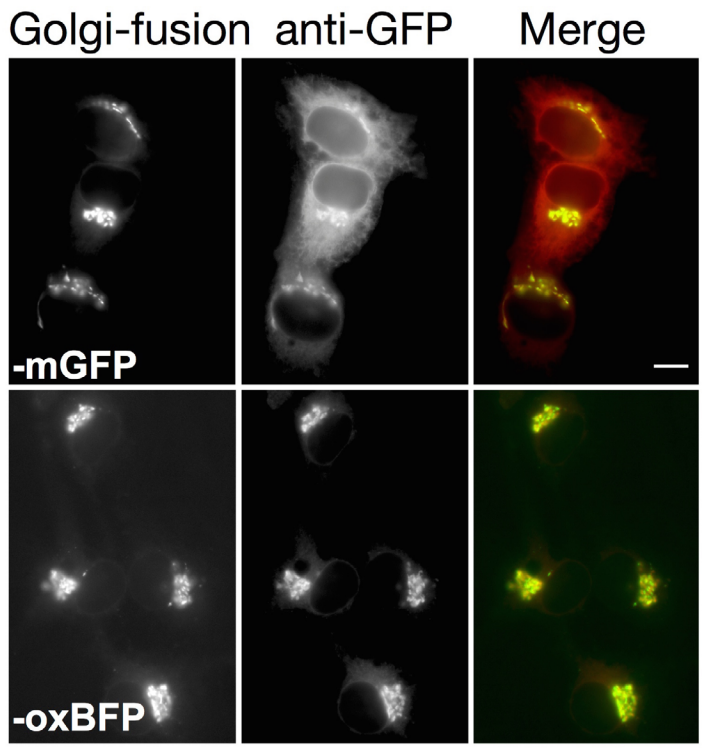

Figure 2. Real world consequences of misfolded FPs in the secretory pathway. A common Golgi complex localized reporter consisting of the signal anchor transmembrane domain of galactosyltransferase was fused to either -mGFP or cysteineless -oxBFP. Cells were fixed, stained with anti-GFP (which recognizes mGFP and oxBFP), and then imaged in the fluorescent protein channel and in the immunofluorescence channel. Both proteins localized to the perinuclear golgi complex, but mGFP has a dark pool in the ER that is revealed by the anti-GFP. The misfolded, mislocalized dark proteins represent a major fraction of the total GalT-mGFP pool. GalT-oxBFP robustly localizes to the Golgi complex.

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ACKNOWLEDGEMENTS AND FINAL WORDS

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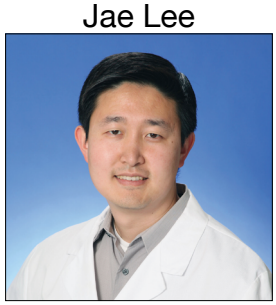

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Mary Gearing

Mary Gearing is a Scientist at Addgene. She got her start as a Science Communications Intern writing for the Addgene blog and website. As a full-time Addgenie, she still enjoys blogging about CRISPR and other cool plasmids! You can follow Mary on Twitter at @megearing.

Benoit Giquel

Benoit Giquel is an Outreach Scientist at Addgene. Based in Europe, he is helping European scientists and institutions share their plasmids via Addgene. When he is not travelling for Addgene, Benoit likes playing the guitar, running, watching sports, and cooking for his family. Follow him on twitter @bengiquel.

ACKNOWLEDGEMENTS AND FINAL WORDS (CONT'D)

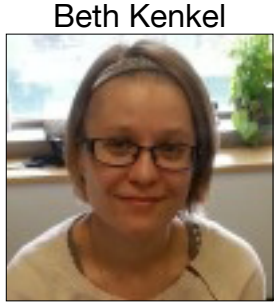
Leila Haery

Leila Haery is a Research Scientist at Addgene and is interested in science education.

A. Max Juchheim

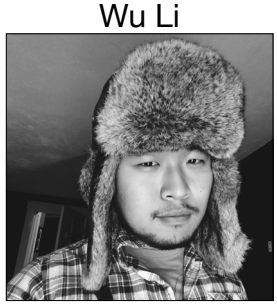
Max is a molecular biologist who does quality control sequence analysis for Addgene. He did his undergraduate studies at MIT where he worked on yeast genetics, and his graduate work at Harvard Medical School, where he studied mouse models of colon cancer. He loves science, but usually prefers to work "behind the scenes", so he's not quite sure how he was roped into writing for Addgene's blog!

Beth Kenkel



Beth Kenkel is currently a research scientist in the Department of Laboratory Medicine at the University of Washington. She is particularly interested in science communication and in vitro diagnostics. Follow Beth on twitter @ElizabethKenkel.

Wu Li



Wu Li is an Outreach Graphic Designer at Addgene with particular interest in branding, motion graphics and UX design.

Caroline LaManna

Caroline is currently the Associate Director of Scientific Outreach at Addgene. She received her Ph.D. in Biomedical Engineering from Boston University. Her goal is to make it easier for scientists to share reagents, communicate their research to others, and teach science in the community.

ACKNOWLEDGEMENTS AND FINAL WORDS (CONT'D)

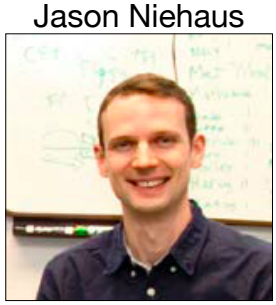
Emma Markham

Emma Markham was previously an Operations Assistant at Addgene where she helped coordinate a variety of efforts across teams at Addgene and participated in European outreach.

Kendall Morgan

Kendall K. Morgan, Ph.D., is a science writer based in North Carolina. She writes about science, health, and medicine for many organizations and publications with the goal to help researchers share their work with each other and the world.

Jason Niehaus

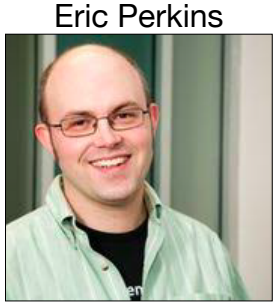


Jason L. Niehaus is the Associate Director of Biology at Addgene and enjoys the intersection of molecular biology and computing.

Marcy Patrick

Marcy is a Senior Scientist at Addgene. She received her Ph.D.in Microbiology and Immunology from the University of Michigan. She loves that her job allows her to interact with scientists from all over the world and help them easily share their reagents.

Eric Perkins



Eric J. Perkins is Addgene's Senior Scientific Project Lead. He used to say his background was in DNA repair, but now that's he's been at Addgene longer than he was in graduate school, he can now say his background is in a little bit of everything.

ACKNOWLEDGEMENTS AND FINAL WORDS (CONT'D)

Julian Taylor-Parker

Julian Taylor-Parker was previously a Scientist at Addgene with a background in molecular medicine and microbiology. He is currently working on earning his MBA with a focus on the health and biotechnology sectors.

Jessica Welch

Jessica Welch was previously an Outreach Scientist at Addgene. She moved to Boston from Australia in 2011 and is now making her way back to Australia. Her interests include microbiology, science education, and helping scientists all over the world share resources.

Lianna Swanson

Lianna graduated from Technion Israel Institute of Technology with a BA in Biology and went on to pursue her Ph.D. at Northwestern University in Evanston, IL. During her graduate school she worked with frogs, yeast, and (mainly) flies. She has extensive molecular biology knowledge and a fair amount of biochemistry experience (bacterial expression, protein purification and western blotting). Lianna is the Director of Biology at Addgene and has been with the organization since 2008.

ACKNOWLEDGEMENTS AND FINAL WORDS (CONT'D)

If you have any questions, comments, or suggestions about how Addgene can improve its educational content, please contact us at blog@addgene.org.

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