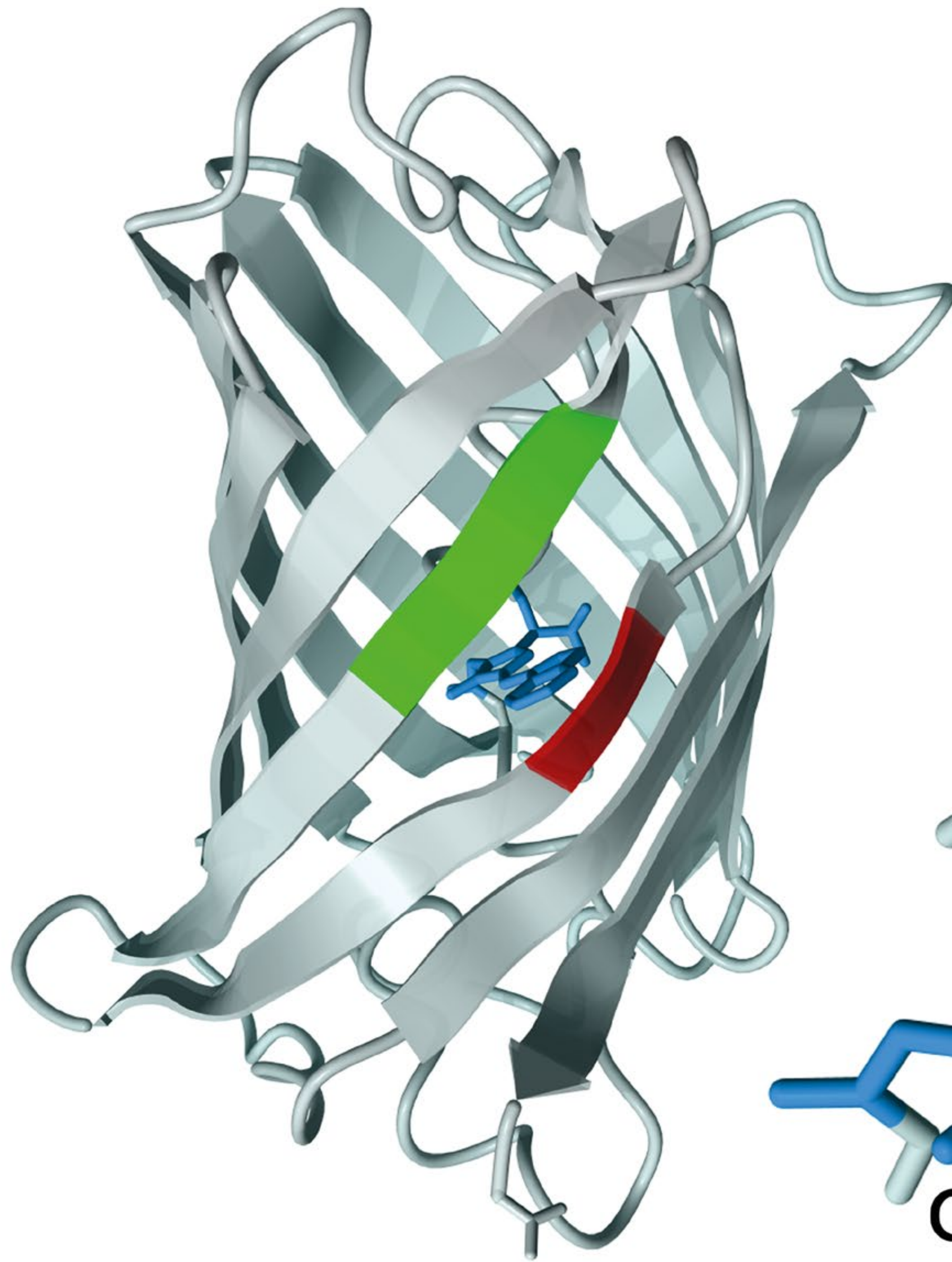
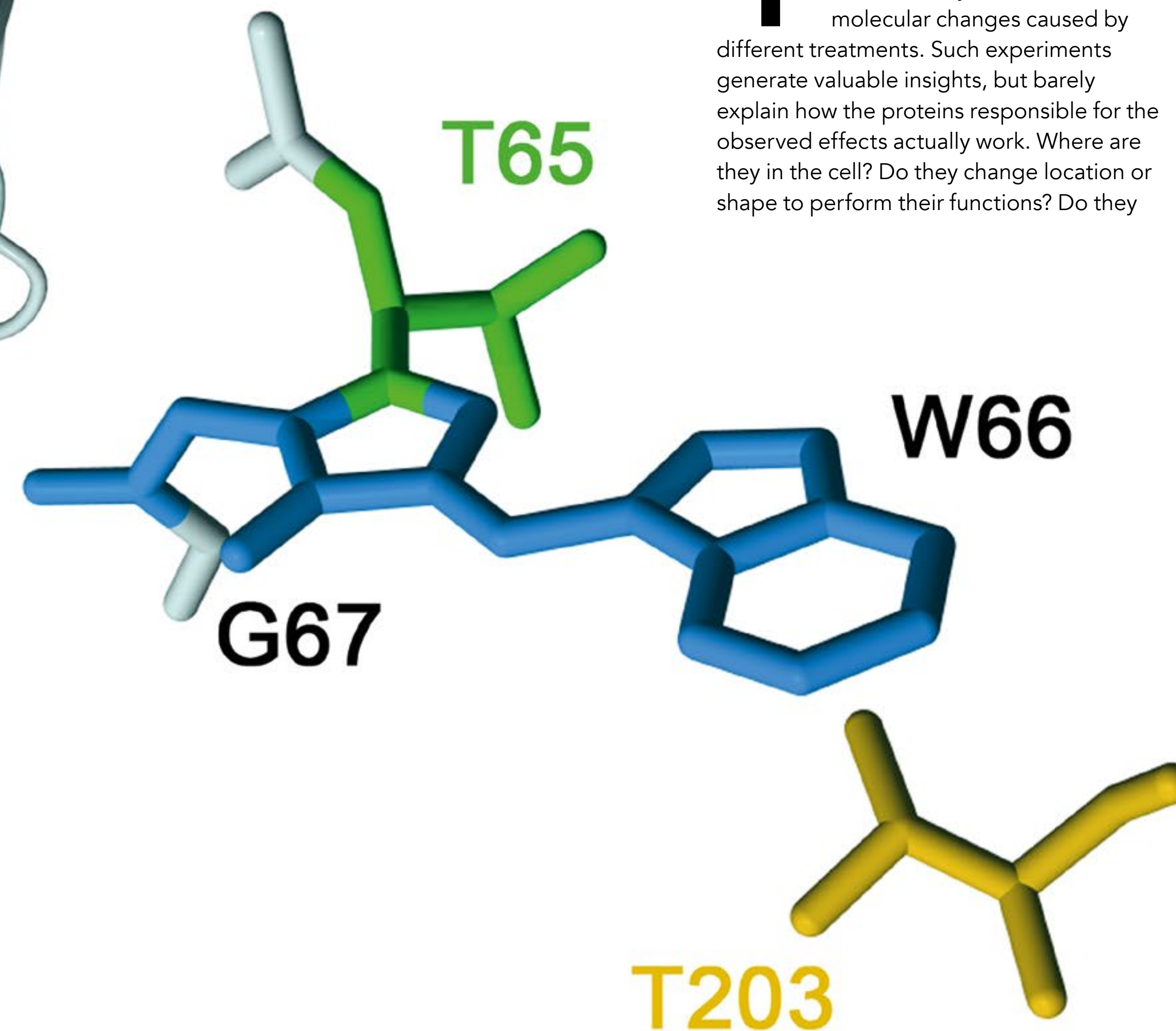




Optimisation of Cerulean A site-directed mutagenesis strategy was employed to optimise Cerulean fluorescence. (A) Residues on β-strand 7 (S147, D148; red), β-strand 8 (L166, I167, R168, H169; green) in the Cerulean X-ray structure (2wso.pdb [27]) were targeted for optimisation by site-directed mutagenesis. The chromophore is coloured blue. (B) T203 (orange) was targeted for optimisation due to its proximity to the chromophore. T65 (green) was also mutated.

Markwardt ML, Kremers G-J, Kraft CA, Ray K, Cranfill PJC, Wilson KA, et al. (2011) An Improved Cerulean Fluorescent Protein with Enhanced Brightness and Reduced Reversible Photoswitching. PLoS ONE 6(3): e17896. doi:10.1371/journal.pone.0017896

Bringing cellular processes to light

The key to understanding how diseases develop or how drugs work is to uncover what goes on inside our cells. However, experimental intervention may perturb normal cell signalling and produce unreliable results. **Dr Mark Rizzo** from the University of Maryland School of Medicine aims to overcome this substantial challenge by applying optimised fluorescent protein tags which interfere minimally with regular cell functions.

To understand cell functions, we often analyse some form of output like a secreted substance, or we destroy cells to detect molecular changes caused by different treatments. Such experiments generate valuable insights, but barely explain how the proteins responsible for the observed effects actually work. Where are they in the cell? Do they change location or shape to perform their functions? Do they interact with other cell components? It is extremely difficult to study proteins in action without disturbing a cell's state.

Dr Mark Rizzo is currently Associate Professor at the University of Maryland School of Medicine in Baltimore. He has set out to develop fluorescent markers that make proteins visible inside the living cell – so-called optical biosensors.

FLUORESCENT PROTEINS

Fluorophores are molecules with the ability to absorb light at a characteristic wavelength and emit it at a longer wavelength. For example, cyan fluorescent proteins (FP) can absorb light from a violet laser with 405nm wavelength and emit blue light at around 480nm. Using genetic manipulation, FP can be connected to other proteins in the cell. Essentially, this creates fluorescent tags that allow us to observe otherwise invisible proteins, using fluorescence microscopy or other suitable means.

Many fluorophores bleach easily during light exposure, which makes long-term experiments impractical. Some are also relatively dim, requiring highly sensitive detection equipment with strong amplification. Dr Rizzo's team has recently modified the molecular structure of the cyan FP 'Cerulean' to improve its fluorescent properties. The result is a variant of this protein, mCerulean3, that is bright and has a lower level of bleaching.

ENERGY TRANSFER

Because the emitted wavelength of cyan FP is still relatively low in the spectrum of visible light, they can be employed as donor fluorophores in Förster resonance energy transfer (FRET) applications. The FRET method is based on the transfer of light energy from one fluorescent molecule to another over very short distances. This effect can be exploited to measure the interaction between fluorophore-coupled proteins or even changes in the shape of single molecules.

Monitoring multiple fluorescent markers at the same time is still a challenge. Depending on the fluorophore combination, FRET technology can produce false positive results. In a quest for reliable multi-colour imaging tools, Dr Rizzo has delved deeply into the physical detail of FP light emission. The insights gained have informed the invention, in collaboration with Dr Jin Zhang at UCSD, of fluorescence anisotropy reporters (FLAREs). As such, Dr Zhang, Dr Rizzo and their teams have already now designed FLAREs in several colours that allow for the imaging of up to three markers at once.

NICHE ENVIRONMENTS

The challenges in designing effective FP go beyond their mere physical properties. A cell contains a variety of micro-environments with distinct biochemical characteristics, such as a low pH or strong oxidising processes. Some FP are not compatible with those extreme conditions and lose their function, or perturb cell processes. In addition, FP may be subjected to regular

> **It is extremely difficult to study proteins in action without disturbing a cell's state. Dr Rizzo has set out to develop fluorescent markers that make proteins visible inside the living cell**

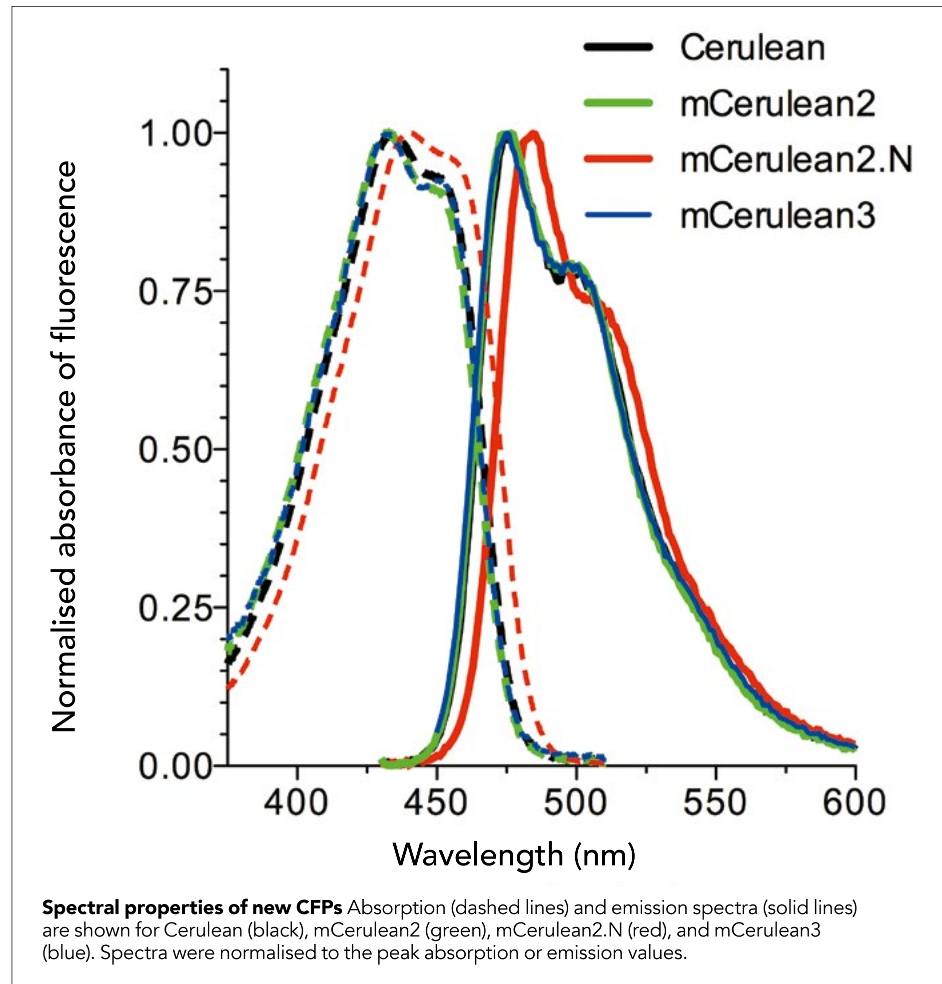
cellular measures of protein formation, transport and quality control. This can interfere with their structure and location in the cell. Altogether, these challenges can lead to unreliable results and artefacts in imaging experiments, restricting the use of many popular FP.

Dr Rizzo and others have been working on the creation of resistant FP which function in diverse cellular micro-environments. Their work has not only expanded the repertoire of available tools, but also yielded significant detailed insights into the molecular changes needed to reduce the susceptibility of existing FP.

CALCIUM SENSORS

Calcium is a vital agent in various cell signalling processes. Dr Rizzo and collaborators have used the FRET method to study the role of calcium in blood vessels of conscious mice using non-invasive fluorescence microscopy. Importantly, while this set-up requires the mice to be sufficiently motionless during imaging, it does not involve pain – hence, anaesthesia is not required. This is a major advantage over studies in the past because it avoids unwanted interference with normal blood pressure regulation by anaesthetics.

The team used transgenic optical biosensor mice which have a genetically encoded FRET-based calcium indicator molecule in their blood vessels. When this molecule is inactive, light energy is transferred from a cyan FP to a yellow FP. This results in detection of the yellow emission. When the sensor molecule is activated by calcium, it undergoes a structural change that creates enough distance between the two FP to



Spectral properties of new CFPs Absorption (dashed lines) and emission spectra (solid lines) are shown for Cerulean (black), mCerulean2 (green), mCerulean2.N (red), and mCerulean3 (blue). Spectra were normalised to the peak absorption or emission values.

Dr Rizzo's achievements have delivered improved versions of several fluorescent proteins, in terms of both their physical properties and usability across various cellular micro-environments

Fluorescence properties of CFPs

Protein	Excitation maximum [nm]	Emission Maximum [nm]	ε_{peak} ($M^{-1}cm^{-1}$)	QY	Brightness[a]	Fluorescence Decay $t_{0.5}$[b] (s)	k_{fold}[c] (10^{-2} s^{-1})	τ[d] (ns)(χ^2 [e])
Cerulean	434	475	43,000	0.48	21	58	0.54	3.17 (0.03 (2.70)
mCerulean2	432	474	47,000	0.60	28	25	1.62	3.04 (0.03 (4.00)
mCerulean2.N	440	484	49,000	0.48	24	36	1.79	2.63 (0.03 (3.41)
mCerulean2.N(T65S)	439	481	43,000	0.46	20	–	–	–
mCerulean3	433	475	40,000	0.87	35	1100	1.90	4.10 (0.02 (1.05)
mTurquoise	434	474	34,000	0.84	29	61	1.93	4.04 (0.03 (1.04)

[a]Brightness was calculated as the product of ε_{peak} and QY.
[b]$t_{0.5}$ value of a single exponential fit for fluorescence decay under constant fluorescence illumination at 300 μW/cm^2.
[c]Refolding rate from denatured protein was determined using the method from reference [3].
[d]The fluorescence lifetime time constant (± SD) was obtained from a single-component fit of TCSPC spectroscopy data.
[e]Value reports the goodness of fit for the lifetime data.
doi:10.1371/journal.pone.0017896.t001

http://dx.doi.org/10.1371/journal.pone.0017896.t001

Q&A

Why did you decide to focus on optimising fluorescent proteins (FP) as a research tool?

These reagents are very valuable to us because they can provide unique insights into the behaviours of living cells and organisms. Even so, FPs are imperfect, which at times limits the measurements that we can make using a microscope. The need to overcome these limitations is our motivation.

Are your improved FP variants commercially available to other researchers?

They are indeed available from the addgene.org plasmid repository, but not presently from commercial sources.

Can FRET-based signals be detected with common types of fluorescence microscopes or is specialist equipment required?

Most fluorescence microscopes can be easily set up to measure FRET by insertion of the right optical filters for illumination and detection. Ironically, this is often more difficult on fancier microscopes, such as confocals, that may not have had the right components built into them in the first place. These kinds of instruments generally need to be configured for FRET experiments at the time of purchase.

Can fluorescence imaging replace more traditional molecular techniques (such as Western blot)?

Undoubtedly, fluorescence imaging is a powerful method that is very well suited for certain kinds of experiments like measuring calcium levels in living cells. That said, there will always be a need for complementary methods like Western blots because there are many things that fluorescence imaging isn't good at. Assessing which protein variants are present in a particular tissue, for example, can be much more clearly determined by Western blots.

What plans do you have for your future research?

We are very interested in understanding the connection between diabetes and cardiovascular disease. It is our hope that these two areas of investigation will merge as the technology allows. We are also very interested in understanding how the nervous system regulates islet function and blood flow. On the technical side, there is also continued interest in fluorescent protein development and improving our microscopy approach for quantitative *in vivo* imaging.

break the FRET effect. This leads to the detection of the cyan emission, because the yellow FP no longer receives any stimulating energy.

The team found that calcium concentrations in blood vessels were indeed much more representative of the normal state, as compared to anaesthesia, and the set-up did not appear to cause stress in mice. In conclusion, using these biosensor mice in an experimental set-up with minimal discomfort provides a basis for more complex studies of the cardiovascular system in the future.

Dr Rizzo's other research interests include the regulation of blood sugar, a key factor in diabetes. His team used a further improved variant of the above-mentioned cyan FP mCerulean3 to study the role of calcium in the signalling within pancreatic beta cells (the cells responsible for blood sugar regulation). Their work has revealed previously unknown details about calcium-dependent processes in these specialised cells.

The achievements of Dr Rizzo and his collaborators have delivered improved versions of several FP in terms of both their physical properties and usability across various cellular micro-environments. These refined biological markers have tremendous potential to give researchers unprecedented insights into the intricacies of cell signalling in any tissue.

Detail

RESEARCH OBJECTIVES

Dr Rizzo's research utilises fluorescent proteins to analyse biological interactions and mechanisms. His biosensor technology, based on FRET-based probes, can be applied to numerous biological problems, and his research has included studies into adipocytes, skeletal muscle and smooth muscle.

FUNDING

National Institutes of Health (NIH)

COLLABORATORS

- Jin Zhang, University of California, San Diego
- W. Gil Wier, University of Arizona
- Erik Snapp, Howard Hughes Medical Institute

BIO

Dr Rizzo received a bachelor's degree in Biochemistry before completing a PhD in Molecular Pharmacology. He received post-doctoral training in Molecular Physiology and Biophysics at Vanderbilt University until 2005, when he joined the Department of Physiology at the University of Maryland School of Medicine.

CONTACT

Mark A. Rizzo, PhD
Associate Professor
Department of Physiology
University of Maryland School of Medicine
655 W Baltimore S
Baltimore, MD 21201
USA

T: +1 410-706-2421
E: mrizzo@som.umaryland.edu
W: https://sites.google.com/site/rizzolabmaryland/