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 fluorescent 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

**Optimisation of Cerulean**

A site-directed mutagenesis strategy was employed to optimise Cerulean fluorescence. (A) Residues on β-strand 7 (D148; red), β-strand 8 (L166, I167, R168, H169; green) in the Cerulean X-ray structure (2wso.pdb) 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.


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.
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. The team used transgenic optical bio sensor 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 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. It implies that calcium, transport and quality control. This 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.

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-dependence 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.