IMAGING AND VISUALIZATION

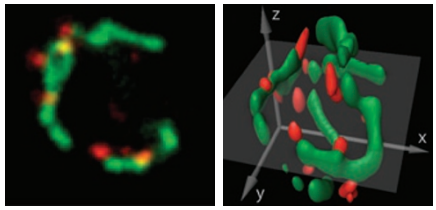
New twists on photoswitchable proteins

Fluorescent proteins with new photoswitching properties allow multilabel imaging at a single detection wavelength and dual-color superresolution microscopy.

With the discovery of GFP many years ago, a green glow seemed to settle over much of biology, but it was not long before additional fluorescent proteins were reported. These now extend over practically the entire visual spectrum.

The properties of these useful tools are constantly being optimized. Fluorescent proteins that can be reversibly switched between on and off states have been reported in the past years. Stefan Jakobs and colleagues at the Max Planck Institute, Göttingen, Germany, now present reversibly switchable fluorescent proteins (RSFPs) with new switching characteristics (Andresen *et al.*, 2008).

All applicable RSFPs until now have been emitters of green or blue-green fluorescence and follow a negative photoswitching mode: the wavelength of light that excites the fluorescence also switches the protein from an on state to a nonfluorescent off state. Jakobs and colleagues, by mutagenizing the photoswitchable protein rsFastLime (a version of Dronpa), identified a variant with the opposite switching mode.



A single confocal section (left) and a three-dimensional reconstruction (right) of live yeast imaged using switching to distinguish between labels. Green, rsFastLime; red, Padron fused to Abp1. Reprinted from *Nature Biotechnology*.

Irradiation of Dronpa or of rsFastLime with blue light results in green fluorescence and concurrent conversion of the protein to the off state; the proteins must be irradiated with UV light to return them to the on state. In contrast, the new protein, which the researchers named Padron, is induced to fluoresce and is also switched on by blue light but is switched off when it is irradiated with UV light. In a separate report, Jakobs and colleagues also report the derivation of monomeric reversibly switchable red fluorescent proteins that show both positive and negative switching modes (Stiel *et al.*, 2008).

"Padron opens up a number of new possibilities" says Jakobs. First, it could be used together with Dronpa (or its variants) to image both proteins at a single detection wavelength. Although the proteins have very similar emissions, their distinct switching modes may be used to image them sequen-

PROTEOMICS

PHOSPHORYLATION AND THE CELL CYCLE

Two groups used quantitative mass spectrometry to look at changes in protein phosphorylation across the cell cycle.

The role of phosphorylation in regulating protein function and cellular signaling is perhaps one of the most important questions in biology. Classical methods like mobility-shift assays allow researchers to identify whether a protein was phosphorylated under certain conditions, but it is extremely difficult to look at large-scale phosphorylation changes across the cell.

With advances in mass spectrometry–based proteomics, it is now possible to obtain detailed information about which proteins and even which specific residues get phosphorylated. It is also possible to obtain quantitative information about changes in phosphorylation stoichiometry. Two independent groups, led by Steven Gygi of Harvard University Medical School and Henrik Daub of the Max Planck Institute of Biochemistry in Martinsried, Germany now report large-scale phosphoproteomic analyses across the cell cycle (Dephoure *et al.*, 2008; Daub *et al.*, 2008).

Gygi's group looked at phosphorylation changes across the proteome in the mitotic and G_1 phases of the HeLa cell cycle, using stable isotope metabolic labeling to enable quantitative analysis. They enriched for phosphopeptides using strong cation exchange chromatography followed by immobilized metal ion affinity chromatography or by using a TiO_2 resin. By pooling the fractions and subjecting them to high mass accuracy liquid chromatography–tandem mass spectrometry (LC-MS/MS), they identified 14,265 different phosphorylation events on 3,682 proteins.

"There are two ways to really regulate something: one, you might modify it by phosphorylation or [another post-translational modification], and the other way would be just to destroy the protein," explains Gygi. His group measured relative phosphopeptide as well protein levels, and were surprised to find that although the levels of most proteins did not change from G_1 to M phases, a huge wave of phosphorylation accompanied mitosis. "We were absolutely amazed; there were at least 1,000 proteins that had increased mitotic phosphorylation in our hands. The big question is whether or not these represent functional phosphorylations or whether they represent some really active kinases that happen to phosphorylate off target," says Gygi, noting challenges that lie ahead in assigning biological function to these sites. Notably, in addition to finding known key regulators, they discovered two new phosphorylation motifs, providing evidence that at least two previously unknown kinases appear to be highly active in mitosis.

Daub's group was specifically interested in what was happening to the phosphorylation status of protein kinases in the M and S phases of the HeLa cell cycle. Kinases are known to modulate each others' activities via phosphorylation or even by autophosphorylation. Daub's group also used stable-isotope labeling, phosphopeptide enrichment and LC-MS/MS, but they added an extra chromatographic step to enrich for kinases themselves by capturing them on immobilized broad-specificity kinase inhibitors. "Most of the kinases in the human kinome are low in abundance, and with the current technologies in

tially, allowing multilabel imaging at a single wavelength and thus avoiding chromatic aberrations.

To demonstrate this, the researchers targeted rsFastLime to mitochondria in live budding yeast and labeled the actin-binding protein Abp1, which localizes to cortical actin patches, with Padron. Using an iterative switching protocol, they monitored the dynamics of these cellular features in three dimensions over several hours. Although the two labels are both green fluorescent proteins, they could be distinguished based on their distinct switching behavior.

Second, the switching of RFSPs can be exploited for subdiffraction-resolution microscopy. Using another new version of Dronpa, so-called broad-spectrum (bs)Dronpa, Jakobs and colleagues demonstrate the utility of these proteins for dual-color superresolution fluorescence microscopy.

"I think we are just at the beginning, with these switchable proteins," says Jakobs. "I would expect that we will see many further developments, more colors and new properties, and these will make applications possible that we haven't even thought about yet."

Natalie de Souza

RESEARCH PAPERS

Andresen, M. *et al.* Photoswitchable fluorescent proteins enable monochromatic multilabel imaging and dual color fluorescence nanoscopy. *Nat. Biotechnol.* **26**, 1035–1040 (2008).

Stiel, A.C. *et al.* Generation of monomeric reversibly switchable red fluorescent proteins for far-field fluorescence nanoscopy. *Biophys. J.* **95**, 2989–2997 (2008).

proteomics we cannot completely cover the whole proteome yet," says Jesper Olsen, second author on the paper.

Daub's group also observed substantial upregulation of phosphorylation in mitotic cells. Phosphorylation of about 75% of the protein kinases they identified was upregulated more than twofold in M phase, suggesting that cell-cycle progression involves extensive rearrangements of signaling networks. In total, they identified 219 kinases containing 1,182 phosphorylation sites, including several new phosphorylation sites not previously implicated in mitosis. However, Gygi's study identified 214 kinases containing 897 phosphorylation sites, demonstrating that in–depth coverage could be achieved without applying specific kinase enrichment approaches.

Both groups attribute their success to careful phosphopeptide enrichment strategies, but also to simply starting with a large sample. "The number one obstacle we overcame was just by using enough protein to start out," says Gygi. "I think that most people know that secret now." A burning question in the phosphoproteomics field, however, is how can we identify every phosphopeptide, and how do we know when this has been achieved? This is extremely difficult to answer, but Olsen anticipates that advances in mass spectrometry will push the detection limits. He says: "Improved dynamic range and faster sequencing speed—I think that's really what we need to have better coverage."

Allison Doerr

RESEARCH PAPERS

Dephoure, N. *et al.* A quantitative atlas of mitotic phosphorylation. *Proc. Natl. Acad. Sci. USA* **105**, 10762–10767 (2008).

Daub, H. *et al.* Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol. Cell* **31**, 438–448 (2008).

NEWS IN BRIEF

PROTEOMICS

Mapping proteolytic events in apoptosis

Current platforms for studying proteolysis have not generally allowed researchers to obtain a complete picture of the cleaved fragments of protease substrates on a large scale. Dix *et al.* now present an algorithm that integrates data from electrophoresis and quantitative liquid chromatography–tandem mass spectrometry analyses of fragment peptides into a 'peptograph', which plots a complete sequence map. They used this method to map caspase-mediated proteolytic events in apoptosis.

Dix, M.M. *et al. Cell* **134**, 679–691 (2008).

BIOINFORMATICS

An atlas of mouse embryo morphology

Petiet *et al.* describe a high-resolution, magnetic resonance microscopy (MRM)-based atlas of normal, transgenic and mutant mouse model morphology at the embryonic and neonatal stages. The database of three-dimensional MRM images with more than 200 annotated structures is freely available at http://www.civm.duhs.duke.edu/devatlas/index.html and should be a useful resource for developmental studies.

Petiet, A.E. *et al. Proc. Natl. Acad. Sci. USA* **105**, 12331–12336 (2008).

GENOMICS

Quality control in next-generation sequencing

It is a challenge to assemble the millions of short sequence reads generated by next-generation sequencers such as the Applied Biosystems SOLiD and the Illumina Genetic Analyzer. Li *et al.* now present 'mapping and assembling with quality' (MAQ), a freely available software tool that uses a mapping quality score to improve alignment accuracy. The tool should be especially useful for detecting single-nucleotide polymorphisms and insertion-deletions.

Li, H. *et al. Genome Res.*, published online 19 August 2008.

IMMUNOCHEMISTRY

Polyubiquitin linkage–specific antibodies

The fate of a polyubiquitinated protein is thought to depend on the lysine residue of ubiquitin through which the chains are linked. To investigate this question more thoroughly, Newton *et al.* developed polyubiquitin linkage–specific antibodies, which recognize two of the best-characterized linkages, via Lys48 or Lys63. These antibodies can be used in different applications, including immunoprecipitation, immunofluorescence, immunohistochemistry and western blotting.

Newton, K. *et al. Cell* **134**, 668–678 (2008).

BIOINFORMATICS

A NetPhorest of phosphorylation motifs

Miller *et al.* present NetPhorest, a valuable database of protein phosphorylation consensus sequence motifs that are recognized by protein kinases and phosphorylation-dependent binding domains. This publicly available atlas (http://netphorest.info/) is fed by an automated data pipeline: these data are mapped to phylogenetic trees to derive linear motif models.

Miller, M.L. *et al. Sci. Signal.* **1**, ra2 (2008).