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MICROSCOPY

Colorful electron microscopy

A multicolor approach specifically labels multiple targets in electron microscopy images.

Electron microscopy (EM) is a powerful imaging modality for providing extremely high resolution images of cellular ultrastructure. However, most biological samples have low endogenous contrast in EM, and identifying subcellular targets using exogenous contrast agents can be challenging, especially when imaging multiple targets in one specimen.

Correlative light and EM approaches such as those that combine EM and super-resolution microscopy are beginning to minimize these issues; however, these methods are still maturing, and better tools are needed. To address this challenge, Mark Ellisman and the late Roger Tsien at the University of California, San Diego and their research teams developed a strategy that allows the positions of multiple targets to be visualized as multicolor EM images.

Their method uses a reagent called diaminobenzidine (DAB) that is widely used for marking specific subcellular targets in EM images. DAB can be oxidized *in situ* by photosensitizing dyes conjugated to antibodies or genetically encoded tags such as miniSOG and horse radish peroxidase to form precipitates. In the case of photosensitizing dyes and miniSOG, the precipitation is triggered by illuminating the sample with specific wavelengths of light. The DAB precipitate reacts with osmium tetroxide and provides strong contrast in EM images, marking the location of the labeled target. In this work, the use of DAB was extended based on the observation that DAB can be conjugated to a lanthanide to generate local deposits of specific lanthanide ions.

In other words, one can label two or more proteins of interest with different tags that catalyze DAB polymerization. In a first round, DAB conjugated to one lanthanide can be precipitated at the site of the first target using a specific wavelength of light for activation. The unreacted DAB can be washed out and replaced with DAB conjugated to a different lanthanide, and precipitation at the site of the second target can be triggered with a different wavelength of light. This process leaves the sites of the different targets marked by the deposition of different

STRUCTURAL BIOLOGY

BLEND-AND-SHOOT CRYSTALLOGRAPHY

A rapid mix-and-inject serial femtosecond crystallography approach enables structure determination of ligand-binding intermediates.

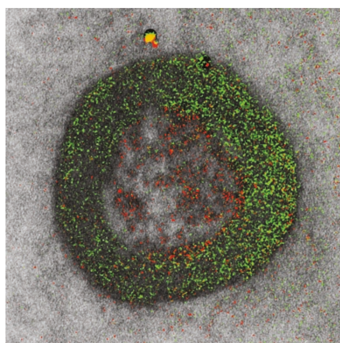
X-ray free-electron lasers (XFELs) are living up to their promise to be a disruptive technology for structural biology. Such lasers are ultrabright and ultrafast, producing femtosecond X-ray pulses that enable a method known as serial femtosecond crystallography. In this approach, a stream of tiny micron- or even nanometer-sized crystals intersects with the XFEL beam, generating single-crystal diffraction snapshots that are merged and averaged to reconstruct a three-dimensional macromolecular structure.

What is really exciting about XFELs, however, is their ability for capturing the dynamics of macromolecular structures. “Molecular systems, like anything else in the universe, change and evolve with time,” notes Yun-Xing Wang of the National Cancer Institute in the US. “In order to understand molecular events, one has to be able to see these events in a sequential time frame.”

By taking advantage of the superior pulse speeds of XFELs, several recent studies have captured macromolecular intermediate structures following reactions triggered by light. But using anything but light as a reaction trigger has remained a challenge.

In recent work, Wang, along with a large team of colleagues, devised a method to capture four structures of the seventy-one-nucleotide adenine riboswitch aptamer domain during the course of adenine ligand binding. Riboswitches are mRNA elements that regulate gene expression via the binding of an appropriate ligand to their aptamer domain. Wang and his colleagues wanted to follow how the aptamer domain structure changes upon ligand binding in order to really understand the mechanism of the regulatory process.

To do so, they used a relatively simple device consisting of a T-junction mixer coupled with a liquid jet. This allowed them to rapidly mix microcrystals of the aptamer domain with the adenine ligand, then inject the crystals into the XFEL beam path for serial



Multicolor EM image of two proteins on the cytoplasmic face of the mammalian endosome. Figure reprinted from Adams *et al.* (2016) with permission from Elsevier.

lanthanides, which are then read out by electron energy-loss spectroscopy (EELS). This is implemented by energy-filtered transmission EM, which ultimately results in pseudocolors representing the different lanthanide positions overlaid onto the traditional black-and-white EM image.

Stephen Adams, a senior research scientist in Roger Tsien's laboratory, recalls that getting the method to work robustly was challenging. Adams notes that "many lanthanide conjugates were synthesized but failed to precipitate on oxidation in cells." The researchers also had to minimize crosstalk between labeling rounds. In addition, they had to improve their data collection with state-of-the-art solid state detectors to capture the relatively weak EELS signals. For researchers hoping to try the method, Adams points to the NIH National Center for Microscopy and Imaging Research (NCMIR) run by Ellisman for collaboration and assistance.

Adams also recalls that this work was one of Tsien's "'Christmas Projects'—a two-to-three-week break at the bench from his email and phone in 2003" as part of a goal "to bring color to electron microscopy as he did to light microscopy with fluorescent proteins." True to Tsien's interests, future research will be aimed at testing a hypothesis proposed by Tsien in 2013 about how long-term memories are stored in the brain.

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Adams, S.R. *et al.* Multicolor electron microscopy for simultaneous visualization of multiple molecular species. *Cell Chem. Biol.* **23**, 1417–1427 (2016).

femtosecond crystallography analysis. The researchers solved four structures of the aptamer domain: two distinct apo states, a ligand-bound intermediate state, and the final ligand-bound state.

Importantly, the mixing and injecting times had to be faster than the conformational transition to the intermediate state. The researchers thus had to carefully consider the kinetics of ligand binding in designing the timing of their experiment. Wang notes that, given the extremely limited XFEL beam time available at the Linac Coherent Light Source facility, where these experiments were done, there was no room for error.

Their real key to success was the small size of the crystals they used. Microcrystals are small enough to allow a ligand to diffuse rapidly throughout the crystal lattice, faster than the timescale of the macromolecular conformational changes. Intriguingly, the researchers observed a phase transition in the crystals, likely a result of the large conformational change in the aptamer domain. The small total volume of microcrystals likely helped minimize the strain created by this transition, which could damage the fragile lattice.

Wang believes that the method will be applicable to many other biomolecular reactions or interactions, at least in principle, if microcrystals or nanocrystals are available. The use of serial femtosecond crystallography, in which diffraction snapshots of individual crystals are collected, will allow both irreversible and reversible processes to be studied. Of course, there are many considerations. "Specific factors that may play a role in the applicability to a given system may include the dynamics and conformational flexibility of the macromolecule, lattice restraints, the degree of conformational changes that take place, and the time-scale of the reaction *in crystallo*," says Wang. It will certainly be interesting to see how other groups build on this proof-of-principle work to learn new things about how macromolecular systems function.

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Stagno, J.R. *et al.* Structures of riboswitch RNA reaction states by mix-and-inject XFEL serial crystallography. *Nature* http://dx.doi.org/10.1038/nature20599 (2016).

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