New Molecular Tools for Light and Electron Microscopy

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Fluorescent proteins (FPs) are invaluable tools for biology, enabling tracking of gene expression, cell fate, and genetically encoded fusion proteins for precise localization within a cell. Traditional FPs developed from jellyfish and coral are limited in wavelengths, consume O_2, and produce a stoichiometric amount of H_2O_2 upon chromophore formation, thus requiring an aerobic environment tolerant of reactive oxygen species. Far-red/near-infrared FPs are desirable for imaging in living animals because less light is scattered or absorbed or reemitted by endogenous biomolecules. Previous near-infrared FPs were engineered from nonfluorescent phytochrome precursors and have had poor quantum yield (QY). We have developed a new class of FP by evolving an allophycocyanin α-subunit from a cyanobacterium, *Trichodesium erythraeum*. Native allophycocyanin is a highly fluorescent hexamer composed of three α+β dimers and uses an auxiliary protein, known as a lyase, to incorporate phycocyanobilin (PCB). The new FP, named small Ultra-Red FP (smURFP), was engineered to bind biliverdin (BV), an endogenous heme metabolite ubiquitous to mammals, without an auxiliary lyase or autoxidation chemistry. It is a dimer of 15 kDa subunits or a tandem dimer of 32 kDa, and has excitation and emission maxima at 642 and 666 nm and the largest QY (0.18), BV incorporation rate, metabolic stability, and photostability of any BV binding FP so far. SmURFP is even more photostable than GFP or Cy5. Collaborations are currently underway to utilize smURFP for superresolution imaging. SmURFP expressed in HT1080 mouse xenografts show significant, visible fluorescence without exogenous BV, but provision of extra chromophore by various means increases the fluorescence yet further. Using smURFP and a phytochrome FP, a far-red/near-infrared fluorescent ubiquitination cell cycle indicator (FUCCI) was created, which should be suitable for monitoring cell cycle progression in intact mammals. The development of this new class of FP and far-red/near-infrared biosensors should dramatically increase our ability to image and monitor dynamics deep in tissues of living animals.

Electron microscopy (EM) achieves the highest spatial resolution in protein localization and has long been the main technique to image cell structures with nanometer resolution. However, making specific molecules standout for EM is a challenge. Recently, powerful genetically-encoded tags have been introduced that allow specific proteins to be tracked by EM via genetic fusion, in a manner similar to how green fluorescent protein (GFP) is used to track proteins by light microscopy (LM). Tagged proteins are revealed by tag-mediated conversion of 3,3′-diaminobenzidine (DAB) into a localized osmiophilic polymer that is readily distinguished under the electron microscope. Currently available EM tags precipitate DAB either enzymatically through peroxidase activity or via photo-generated singlet oxygen. While such tags are powerful tools for "painting" individual proteins, researchers lack analogous tools for marking biochemical processes, or non-proteinaceous molecular species for EM. To complement the existing EM tags, we describe "Click-EM," a new method for imaging nucleic acids, lipids, and glycans via bio-orthogonal ligation of photo-sensitizing dyes to functionalized metabolic analogs. These analogs

mimic the fates of their natural counterparts and can be used to track cellular metabolism. Analogs functionalized with azides and alkynes can be selectively ligated to chemical probes that do not react with endogenous (unlabeled) biomolecules. For detection, azide- and alkyne-functionalized analogs can be revealed by Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), a reaction often referred to as "click chemistry," to appropriately functionalized dyes. These labeled structures, conjugated to a singlet oxygen-generating fluorescent dye (a "photosensitizer"), can be visualized first by fluorescence and subsequently by EM through photogeneration of singlet oxygen for DAB precipitation. Using these methods, we have imaged neuronal protein assemblies within the full context of cellular ultrastructure and have visualized DNA replication (see Fig. 1) and mRNA transcription at the nanometer scale.

Distinguishing multiple biomolecules in EM is presently limited to attachment of different sizes of gold particles or quantum dots to specific antibodies, which poorly penetrate into the strongly fixed cells or tissue required for optimal cellular ultrastructure. Localized precipitation of DAB by antibody conjugates or genetically-encoded chimeras with photosensitizers and subsequent staining with osmium can overcome many of these constraints but is limited to a single protein or tracer "color". For "multi-color" EM, we have now synthesized "Ce-DAB" and "Pr-DAB", shorthand for conjugates of DAB with chelates of Ce or Pr. Ce-DAB is locally photooxidized with the first photosensitizer tracer, and then the polymer is quenched. Pr-DAB is either oxidized by a peroxidase-antibody conjugate to a second label, or photooxidized with a second targeted photosensitizer irradiated at much longer wavelengths than the first. The two deposited lanthanides can be detected by electron energy loss spectroscopy (EELS), selectively imaged with energy-filtered transmission EM, and overlaid as elemental maps on a conventional electron micrograph. Pancreatic cancer cells labeled with NBD-ceramide and an EpCAM antibody gave the expected Golgi and plasma membrane staining respectively. We detect sharing of a single synapse by two adjacent astrocytes in mouse brain slices and reveal the endosomal localization of cell-penetrating peptides in tissue culture cells, demonstrating high spatial resolution and selectivity.
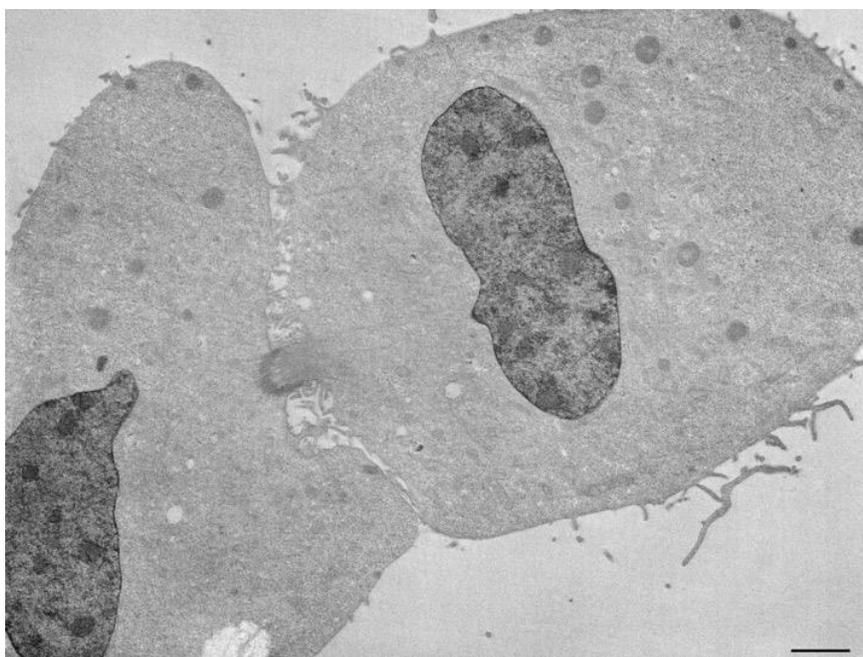
Fig. 1. Click-EM imaging of newly replicated DNA in a dividing HeLa cell. Live cells were pulsed for 12 hr with 5-ethynyl-2′-deoxyuridine (EdU), a nucleoside analog readily incorporated into DNA during replication. After fixation with glutaraldehyde, alkyne-containing DNA was conjugated to azidodibromofluorescein (DBF) via CuAAC. Cells were incubated with DAB and illuminated for 5 min with blue light for singlet oxygen generation and photo-oxidation of DAB, which formed optically dense precipitates coincident with DBF fluorescence. Cells were stained with OsO_4, embedded in resin, and thin sectioned for EM. Scale bar 2 μm.