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Bridging the Mesoscale Gap

The precise location of Golgi membranes and resident proteins during mitosis remains unclear, partly due to limitations of molecular markers and the resolution of light microscopy. We targeted a fluorescent protein and tetracysteine (TC) motif to the Golgi lumen by fusion to the first 117 residues of α -mannosidase II. The TC can be acutely reduced with a membrane-permeant phosphine, labeled with ReAsH, monitored in the light microscope, and used to trigger the photoconversion of diaminobenzidine (DAB), allowing 4D optical recording on live cells and correlated ultrastructural analysis by electron microscopy (EM). These methods reveal that Golgi reassembly is preceded by the formation of four co-linear clusters at telophase, two per daughter cell. Within each daughter, the smaller cluster near the midbody gradually migrates to rejoin the major cluster on the far side of the nucleus and asymmetrically reconstitutes a single Golgi, first in one daughter cell and then in the other (Gaietta *et al.*, *PNAS* 2006).

A new membrane-permeant biotin conjugate of FAsH provides an alternative means by which a short genetically-encoded sequence (now a TC) can be biotinylated inside live cells. Thus TCs can now be detected by streptavidin-conjugated labels such as quantum dots.

A single GFP can incorporate both a TC inserted after residue 173 and a TC appended to either N- or C-terminus and be labeled with two ReAsH molecules, doubling the brightness without significant dye-dye quenching. The efficiency of photoconversion driven by FRET from the GFP is also improved, which should increase the sensitivity of correlated EM. The TC inserted at 173 is also particularly resistant to palmitoylation.

During selection for optimized biarsenical-binding TCs, one novel tag, YRECCPGCCMWR-GFP, was discovered capable of inducing photo-reversible self-aggregation upon biarsenical labeling in living cells or *in vitro*. Using this tag, we obtain new insights to the mechanism of PKA activation in living cells. Fusion of the Biarsenical Aggregating GFP (BA-GFP) to the regulatory (R) or catalytic (C) subunit of PKA led to dye-dependent, photo-reversible self-aggregation capable of trapping and silencing PKA holoenzyme in compact fluorescent puncta. While the classical model of PKA activation states that elevated cAMP alone is sufficient for dissociation of the catalytic subunit from the regulatory subunit of PKA, we find that RI α and C α co-aggregate even in the presence of elevated cAMP, and dissociate only in the presence of both elevated cAMP and substrate. Conversely, cells weakly expressing RII α release C α upon elevated cAMP alone, dependent on the auto-phosphorylation site in the RII α inhibitory domain. Therefore, both RI α and RII α require elevated cAMP and substrate for C α dissociation, but RII α is its own substrate (Martin *et al.*, submitted for publication).

We have engineered Green Fluorescent Protein mutants (sosGFPs) that respond to singlet oxygen with an irreversible increase in their 490 nm: 400 nm excitation ratio. These mutants can

be used to assay the proximity of protein X to protein Y by fusing X to a TC, staining with ReAsH, illuminating ReAsH at 568 nm, and intermittently checking the 490/400 nm excitation ratio of sosGFP (emitting at 510 nm) fused to protein Y to see how fast the excitation ratio change develops. The closer X and Y bring the singlet oxygen generator and sensor, the more rapidly the sensor (sosGFP) will respond to illumination of the generator. Singlet oxygen transfer (SOT) has a distance range (~100 nm) at least 10X greater than that of FRET (6-8 nm) and lacks FRET's dependence on chromophore orientation. On the other hand, FRET is faster and reversible. To demonstrate SOT, we constructed gap junctions fused on one cytoplasmic side to sosGFP and in the adjoining cell to cytoplasmic TC. From the known dimensions of gap junctions, the TC and sosGFP are ~25 nm apart. Excitation ratio changes were only observed following illumination of heterojunctions stained with ReAsH. FRET from sosGFP back to ReAsH was undetectable, as expected.

To provide an alternative to TCs, we have devised a small fluorescent chelator whose membrane-impermeant complex with nontoxic Zn^{2+} ions binds tightly but reversibly to hexahistidine (His_6) motifs on surface-exposed proteins. This live-cell label resolves a current controversy concerning externalization of the stromal interaction molecule STIM1 upon depletion of Ca^{2+} from the endoplasmic reticulum. Whereas N-terminal fluorescent protein fusions interfere with surface exposure of STIM1, short His_6 tags become accessible to the dye or antibodies, demonstrating externalization (Hauser & Tsien, *PNAS*, 2007).

In a separate set of experiments, we have developed a new technique, which we call Defocused Orientation and Position Imaging (DOPI). We can determine the 3-D orientation of the dye, i.e. the direction of the transition dipole moment, to within $\sim 10^\circ$, in addition to the dye's centroid to nanometer resolution via FIONA. The idea is to defocus the microscope objective lens, which makes the shape of the image sensitive to the dye's orientation. In addition, to optimize signal-to-noise, we have used a dual-view which allows us to simultaneously image DOPI and FIONA. We have applied this to myosin V (Toprak *et al.*, *PNAS* 2006) and recently to myosin VI, using a bis-functional rhodamine dye which is rigidly attached to the calmodulin in the neck region. Myosin V steps between 57° and 128° , consistent with the 36 nm powerstroke. The results for myosin VI are surprising.

In addition, we have developed a new form of our FIONA (Fluorescence Imaging with One Nanometer Accuracy), which does not rely on fluorescence, and is particularly well suited to *in vivo* imaging. It is called bFIONA (bright-Field Imaging with One Nanometer Accuracy). We use bright-field illumination to imaging individual melanosomes (dark pigments in *Xenopus* cells), being moved by myosin V, kinesin-2, and dynein. We get 2 nm accuracy and 1 msec temporal resolution, and can see a melanosome being passes between actin- and microtubule-based motors (Kural *et al.*, *PNAS*, 2007).

Publications

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