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Single-Molecule Fluorophores for Cellular Imaging

This P20 exploratory program brings together experts in synthetic chemistry, biophysics, single-molecule imaging in cells, and developmental microbiology to focus on three key areas: design and synthesis of new fluorophores for improved imaging in cells, photophysical assessment and utilization of new single-molecule fluorophores in cellular targeting schemes, and application of new imaging methodologies to explore mechanisms of regulatory protein localization in *Caulobacter crescentus*.

Design and synthesis efforts in the project have concentrated on the DCDHF family of environmentally sensitive fluorophores. We have examined systematic changes in the amine donor structure and in the π-conjugated system by implementing different combinations of aromatic units (benzene, naphthalene, anthracene, thiophene, pyridine) and alkene units, thus tuning the fluorescence activity across the visible and into the near IR. These fluorophores have been modified and functionalized in various locations (donor amine end, central π-core, acceptor heterocycle end) with a wide variety of groups so as to modify the hydrophobic/hydrophilic/amphiphilic properties and to impart chemical reactivity (via installation of metal chelating groups, maleimide, and hydroxysuccinimide ester, SNAP and tritylated/phosphoramidate groups). These efforts have sometimes required enhancements in basic synthetic methodology to enable the management of multiple functional groups and especially in the case of installation of reactive sites. We have also examined the preparation and characterization of fluorophores with two DCDHF chromophores with controlled spatial relationships so as to examine and take advantage of their excitonic interactions.

Photophysical characterization of the new fluorophores has included measurement of fluorescence quantum yields and excited state lifetimes in a variety of solvents in order to assess environmental sensitivity of the new structures. Separate measurement of single-molecule photobleaching quantum yields (numbers of photons emitted before photobleaching) also allows comparison of the photostabilities of the new fluorophores. In a specific cellular study, seven different amphiphilic forms of the DCDHF fluorophores were utilized as fluorescent lipid analogs, and single-molecule diffusion coefficients were determined in the plasma membrane of CHO cells showing that the new dyes are competitive with the well-known TRITC fluorophore. A second thrust has involved use of the DCDHF dyes as fluorescent tags for observing poly(Arg) cell-penetrating peptides interacting with CHO cells. In a different approach, the DCDHF dyes are being explored as tags for protein fusions based on O6-alkylguanine-DNA-alkyltransferase which has been transfected into a target cell at a location of interest. Exploring these dyes as protein labels has shown that the emission of certain derivatives can dramatically increase upon attachment to a protein. We have also discovered that pairs of DCDHF molecules can form a parallel H dimer structure that gives rise to an absorption blue shift and loss of fluorescence. In this configuration, the DCDHF molecules act both as quencher and as emitter, and a set of molecular beacon oligonucleotides have been prepared that can be used as single-molecule sensors of target DNA.

We have succeeded in visualizing single molecules *in vivo* of two bacterial cytoskeletal proteins: the actin homologue MreB and the tubulin homologue FtsZ. The MreB protein in *Caulobacter crescentus* forms a helix structure along the long axis of the cell. In this case, we have produced MreB-EYFP fusions and have observed the directed motion of single molecules as the spiral structure polymerizes *in vivo*. Different fluorescent-tagged MreB molecules in the spiral structures are observed to move in different

directions. This somewhat surprising observation suggests the structures are spiral bundles of many short MreB filaments without a preferred direction at the MreB actin filament level, although the filaments themselves have their own individual directionality. This ongoing study represents the first single-molecule examination of cytoskeletal proteins in live bacterial cells and the first time molecular "treadmilling" has been observed *in vivo* in bacteria. The motion of the single molecules through the filaments yielded a superresolution image of the filament shape and position to 30 nm. Recently, we have succeeded in achieving polymerization of pure MreB and MreB-EYFP *in vitro*. Significantly, we can now observe the kinetics of fluorescently tagged MreB (MreB-EYFP) polymerization *in vitro* and single-molecule analysis of the *in vitro* polymerization process is in progress. In the FtsZ case, we have succeeded in observing the movement of individual fluorescent-tagged FtsZ tubulin-like molecules within the *Caulobacter* cell division Z-ring.

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