**Final report**

**Genetic encoding and click chemistry for super-resolution imaging of proteins**

**(“GECCCCA”)**

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**A1. Final publishable summary report**

**A1.1. Project title**: Genetic encoding and click chemistry for super-resolution imaging of proteins

**A1.2. Project objectives**

The goal of this proposed work is to develop an efficient, minimally invasive method for site-specific fluorescence labeling of mammalian intracellular proteins, using genetic code expansion in conjunction with an efficient bioorthogonal reaction. The new protein labelling method will be used for super-resolution imaging of cellular proteins, to reveal protein dynamics previously unresolved with traditional protein tagging methods.

**A1.3. Main results, conclusions, and their potential impacts**

We have successfully combined genetic code expansion with inverse-electron-demand Diels-Alder cycloaddition for super-resolution imaging of cellular proteins. We did so in a series of steps. First, we determined that among the two unnatural amino acids—bicyclo[6.1.0]nonyne-lysine (BCNK) and *trans*-cyclooctene-lysine (TCOK)—that can be used to react with tetrazines for Diels-Alder reactions, BCNK is better suited for intracellular protein labeling as it can be cleanly removed from cells via washing, resulting in no/minimal labeling background. In contrast TCOK, despite being a more kinetically efficient handle for Diels-Alder, sticks non-specifically to the cell interior. Second, we optimized the pyrrolysyl tRNA synthetase/tRNACUA expression system via the optimization of Pyl tRNACUA levels, and used the optimized system for efficient intracellular protein production. Third, we identified amino acid positions on β-actin and vimentin which can be replaced with the amber stop codon, and subsequently, BCNK, without affecting their functions. BCNK-incorporated actin and vimentin can be produced efficiently in mammalian cells. Fourth, we confirmed that genetic code expansion in conjunction with Diels-Alder chemistry provides sufficient labeling specificity, by reacting actin and vimentin bearing BCNK with a tetrazine-fluorophore in cells. In-gel fluorescence analysis of the resulting cell lysate showed minimal off-target labeling on the proteome. Upon microscopic imaging, we did observe evidence of off-target labeling, determined the source to be a population of Pyl tRNACUA which is aminoacylated with BCNK but has not been used in protein translation, and devised an empirical solution to overcome this background. Fifth, we identified a suitable fluorophore to be used for intracellular STORM imaging—a recently reported silicon-rhodamine fluorophore developed by the Johnsson Laboratory (EPFL Lausanne). Lastly, we combined discoveries and improvements we have made to STORM imaging of actin and vimentin in mammalian cells. STORM images of these cytoskeletal proteins revealed fine ultrastructural details, including individual vimentin intermediate filaments and different classes of filamentous actin in the cytosol and nuclei of cells.

We expect our labeling technology to have a broad impact on the biological and super-resolution imaging community. Currently, the performance of many super-resolution imaging techniques is limited by the photophysics and size of the fluorescent label. Fluorescent proteins, the most commonly used tool for cellular imaging, are too dim and do not emit enough photons before photobleaching, limiting the achievable image resolution. Small organic fluorophores are known to have much better photophysical properties than their protein counterparts, but common methods to site-specifically introduce organic fluorophores to cellular proteins involve addition of a protein or a peptide tag, all of which can still perturb the function of protein they are fused to. Our work introduces a new labeling strategy that involves replacement of a single amino acid within a protein with an organic fluorophore, a maneuver that is minimally perturbative to protein function. Moreover, unlike when a protein or a peptide tag is used, the label sites are no longer limited to protein termini with our strategy. The flexibility in label placement, upon combining with super-resolution imaging, should allow creative applications of the method, such as topological mapping of macromolecular complexes in cells. Further studies should lead to elucidation of new biological insight unobtainable with previously existing tools.