A Genetically Encoded Tag for Correlated Light and Electron Microscopy of Intact Cells, Tissues, and Organisms

Xiaokun Shu¹,²*, Varda Lev-Ram², Thomas J. Deerinck³, Yingchuan Qi¹,⁴, Ericka B. Ramko⁵, Michael W. Davidson⁵, Yishi Jin¹,⁴, Mark H. Ellisman³,⁶, Roger Y. Tsien¹,²,⁷*

¹Howard Hughes Medical Institute, University of California at San Diego, La Jolla, California, United States of America, ²Department of Pharmacology, University of California at San Diego, La Jolla, California, United States of America, ³National Center for Microscopy and Imaging Research, Center for Research on Biological Systems, University of California at San Diego, La Jolla, California, United States of America, ⁴Division of Biological Science, Section of Neurobiology, University of California at San Diego, La Jolla, California, United States of America, ⁵National High Magnetic Field Laboratory and Department of Biological Science, The Florida State University, Tallahassee, Florida, United States of America, ⁶Department of Neurosciences, University of California at San Diego, La Jolla, California, United States of America, ⁷Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California, United States of America

Speaker: Bo-Hua Chen
Coach: Dr. Wei-Yuan Yang
Sit-in: Dr. Cheng-Chung Wang
awarded the 2008 Nobel Prize in chemistry for his development of the eGFP

invited to speak as an Academia Sinica Lecturer in December 2009

Academician in 2010

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S65T shifted the absorption maximum from 395 to 488 nm and increased fluorescence, photostability

Subcellular localization is important

The dynamic behavior of cells is a consequence of the elaborate interactions between complexes/organelles.

Microscopy to reveal the important information to understand complex cellular function.
Microscopy

Fluorescence light microscopy
- Sample preparation is easy and relatively inexpensive.
- Appropriate tags provides the ability to track specific proteins of interest in living cells.
- Limitation: resolve two objects separated by ~200 nm.
  - Objects that are closer than 200 nm are blurred into a single spot.

Electron microscopy
- EM provides the unique “space” where all objects (labeled and un-labeled) can be visually explored at high resolution.
- You can get up to a hundred-fold higher useful magnification from EM than from light microscopy.
- Ultrastructures: mitochondria, Golgi, lysosome, membrane with high curvature, ….
- Provides nanometer spatial resolution, but is not available in live-cell imaging.
- Lacks good tag to identify protein of interest.

Annu Rev Biophys Biomol Struct. 2006;35:199-224
Identifying the protein in EM

- Immunogold labeling
  - Fixation to preserve the ultrastructure inhibits the diffusion of antibodies and impairs antigenicity
  - Triton-100 facilitating the diffusion of antibodies degrades membrane.
    - Membrane is not vivid
  - High specificity to the cross-linked antigen
    - Expensive
    - Distance from epitope (30nm)
    - Not all targets can be labeled.

LAMP1 localization in human HepG2 cells

Genetically encodable tag

- benefit: produced in cells
- Can GFP be visible to electron microscopy?
  - generation of endogenous singlet oxygen by photoactivated GFP
    Biol Chem. 2000 Dec;381(12):1251-8

- Oxygen radicals generated during the GFP bleaching process can photooxidize DAB into an electron-dense precipitate that can be visualized by routine electron microscopy and electron tomography.
- human Golgi resident glycosylation enzyme, N-acetylgalactosaminytransferase-2 fused to eGFP
ROS generated by illumination can polymerize DAB to a light brown precipitate which can be stained by Osmium and imaged in EM.

Photo-oxidation for EM

Periodic Table of the Elements
Singlet oxygen

Ground state oxygen, $O_2$, is a triplet diradical.

Degeneracy = $2S + 1$

$S$ is the total electron spin angular momentum

$2\left[ \frac{1}{2} + \frac{1}{2} \right] + 1 = 3$

$2\left[ \frac{1}{2} + \left( -\frac{1}{2} \right) \right] + 1 = 1$

Disobey Hund’s Rule

Bond order = $(8 - 4) / 2 = 2$

http://www.meta-synthesis.com/webbook/16_diradical/diradical.html
Genetically encodable tags

- **GFP**
  - $^1\text{O}_2$ quantum yield ($^1\text{O}_2$/photon absorbed) is low and unquantifiable. *Biophys J* **94**: 168–172

- **Tetracysteine-ReAsH system**
  - 12-residue peptide tag
  - $^1\text{O}_2$ quantum yield is 0.024 (the best previous genetically targetable generator)
  - Adding of biarsenical dye ReAsH into cell is required.
    - Deep tissue or organism labeling is difficult.
  - Nonspecific background signal
  - As is toxic
Aim

- engineer fluorescent protein with high efficiency of production of singlet oxygen
Arabidopsis thaliana phototropin 2

- binds to its chromophore, flavin mononuceotide (FMN) very tightly (dissociation constant ~0.1 nM)
  - FMN can generate singlet oxygen efficiently. (quantum yield=0.51)
  - The only cofactor which is necessary for the mitochondrial electron transport chain is thus present in nearly all cells.
- LOV2 domain
- 106 amino acids
  - less than half the size of GFP (248a.a.)

Arabidopsis thaliana phototropin 2

- The excited state energy of FMN is consumed to form a covalent bond with cys426. *J Biol Chem* **276**: 36493–36500
- Mutated Cys426, so excitation energy may be used to produce singlet oxygen.
- Saturation mutagenesis at position 426 and screen for optimal single oxygen production.
  - LOV2 domain was fused to IFP 1.4 (ex 684/ em 708nm)

19 mutants

- C426A
- C426W
- C426K

684nm

488nm

1O2

708nm

684nm
Screen for better $^1\text{O}_2$ generator

- E. coli colonies were imaged before and after 488nm illumination

$\sim$70% decrease $\cdots$ C426G

A

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$\Rightarrow$
MiniSOG

- increase brightness of the C426G mutant
  - Saturation mutagenesis of other residues surrounding the chromophore binding site
  - plus random mutagenesis
- for mini Singlet Oxygen Generator

Predicted structure
MiniSOG

- **Excitation**
  - Fluorescence quantum yield: 0.37
  - 448nm: $(16.7 \pm 0.7) \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$
  - 473nm: $(13.6 \pm 0.5) \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$

- **Emission**
  - 500nm
  - 528nm

- Singlet oxygen quantum yield: 0.47

![Singlet-oxygen fluorescent probe](image)
Size exclusion chromatography of miniSOG

MiniSOG was determined to be monomeric in solution
Successful localizing

miniSOG labeled proteins and organelles appeared to have correct localizations in cultured mammalian cells.
Schematic diagram
Procedure

1. **transfected cells**
   - **fixation**: 2% glutaraldehyde pH 7.4
     - blocking 50mM glycine, 10mM KCN, 5mM aminotriazole (reduce background reaction)
   - **confocal** microscope (identify transfected cell)
   - ice cold DAB solution bubbled with oxygen and freshed
   - illuminate and stop as soon as light brown DAB polymer appear (with FITC filtered light from xenon lamp, 2-10mins)
   - post-fixation with 1% osmium tetroxide 30min on ice
   - stained with 2% uranyl acetate at 4°C (protein, nucleic acid)
   - dehydrated in ethanol and infiltrated by resin

2. 3ug SynCAM2-miniSOG DNA delivered into lateral ventricel of embryos by in utero electroporation
   - p7, p21 brain removed, fixed by perfusion with 4% formaldehyde
   - sliced to 100 μm sections
   - area of interest identified by confocal
   - postfix with 2% glutaraldehyde
   - blocking 50mM glycine, 10mM KCN, 5mM aminotriazole (reduce background reaction)
Procedure

Photooxidized areas of resin-embedded transfected cells or tissue identified by transmit light and sawed out (cut)

↓

Ultra-section (50-70nm)

↓

80keV TEM

Resin embedded H2B-miniSOG expressing cells

↓

0.5μm thick section

↓

400keV Electron tomography
α-Actinin cross-links actin bundles and attaches actin filaments to focal adhesions.

α-actinin-miniSOG image is consistent with published observation.
H2B in nucleus

transmitted light imaging

Fibrillar chromatin structures near the nuclear envelope and nuclear pores were also observable at high resolution.
transmitted light imaging

differential contrast

well-preserved morphology of outer and inner membranes of mitochondria
Cx43 forms gap junction channels

densely packed Cx43 gap junctions

black dots on the outside of trafficking vesicles may represent single connexon

much sparser, more random labeling

six in each hemichannel
C. elegan

targeted to the mitochondria in body wall muscles

miniSOG is under muscle-cell specific promoter
adjacent unlabeled mitochondria in a different cell type
Could miniSOG reveal new molecular details of the organization of neuronal synapses?

SynCAM1-miniSOG was found only at presynaptic terminals, identified by the presence of synaptic vesicles. SynCAM2 localized to postsynaptic sites.
Conclusion

- The utility of miniSOG was demonstrated by correctly labeling several already well-understood proteins in mammalian cells, nematodes and rodents.

- Correlated confocal and EM imaging could be performed with miniSOG, producing excellent EM contrast, efficient labeling, and good preservation of ultrastructure.

- SynCAM1 and SynCAM2 can be precisely indicated by miniSOG to the presynaptic and postsynaptic sides of mammalian brain synapses respectively.
Discussion

- Although the method is limited in that one can tag only one kind of protein at a time, the tag is useful and will be a valued tool for cell biologists.

- MiniSOG will grant new powers to electron microscopy, permitting scientists to pursue answers to questions previously impossible to ask.
Thank you for your attention!