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REVIEW

Advances in molecular probe-based labeling tools and their application to multiscale multimodal correlated microscopies

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Abstract The need to determine the precise subcellular distribution of specific proteins and macromolecular complexes in cells and tissues has been the major driving force behind the development of new molecular-genetic and chemical-labeling approaches applicable to high-resolution, correlated, multidimensional microscopy. This short review is intended to provide an overview of recently developed and widely used electron microscopy (EM)-compatible probes, including tetracysteine tags, *mini singlet oxygen generator* (MiniSOG), *time-specific tag for the age measurement of proteins* (TimeSTAMP) with MiniSOG, and *enhanced ascorbate peroxidase* (APEX). We describe how these highly specific and genetically introduced EM probes are now used, in conjunction with lower resolution light microscopic methods, to obtain wide field or dynamic records of live preparation or of large maps in 3D using recently developed laboratory-scale X-ray microscopes. The article is intended to enable researchers through a high-level view of the toolbox of labels available today for studies aiming to analyze dynamic subcellular and molecular processes in cell culture systems as well as in animal tissues—and ultimately allow investigators to determine the precise location of macromolecular complexes by EM.

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Introduction

Microscopy has played a central role in biomedical research, due in large part to the vast range of scales over which it is capable of delivering information. It is arguably the most important tool in biomedical science. Even today, microscopy continues to experience a tremendous expansion in its application to research, catalyzed in large part by the introduction of genetically encodable fluorescent proteins (FPs) to live-cell imaging [11, 21] and the synergistic development of new instrumentation and imaging modalities, leading to a wealth of new discoveries in cell biology and medicine. Key to the success and widespread adoption of these technologies is their ease of use, robustness, and consistency of results obtained. Efforts to expand, improve, and simplify the use of FP reporters have spawned rapid growth of research and development of new probes systems with broadening impact on basic and biomedical research. A related undertaking involves extending these types of molecular-genetic probes to other forms of microscopy, particularly electron microscopy (EM) (for review, see [8]). The ultimate goal is that, with the greatly improved resolution afforded by EM, these new types of molecular-genetic labels will support advances in quantitative spatial proteomics in small sub-volumes by enabling direct in situ visualization of molecules and macromolecular complexes in cells and tissues. These new types of genetic labels promise to deliver unprecedented in situ spatio-proteomic information in cells and tissues.

All these efforts are meant to impact bioscience, as there is a *general* need to improve the resolution and fidelity of protein labeling and macromolecular recognition achievable by

electron microscopy and improve and streamline methods available for multiscale, multicolor, multimicroscope modality imaging. A current focus in the biology field is to continue to develop and apply molecular probes for correlated imaging to propel multiresolution analysis of the structure and function of proteins in supramolecular complexes underlying cellular processes. The expectation is to enable biomedical researchers to follow small molecular components, requiring a resolution of <10 nm, and support their ability to do supramolecular imaging throughout a continuous field of view as great as 100 μm wide. This range is required to observe and map molecular constituents in their cellular and tissue context.

Despite research efforts to decode entire genomes and recent advances in genomic-screening technologies for uncovering mutations in various genes linked to different forms of diseases, we are often left with a profound gap in understanding their final product of regulation and expression. Particularly in complex organisms, we often lack knowledge of the function and distribution of many cellular proteins. Furthermore, knowing the unique expression dynamics and distribution of each protein from a systems biology point of view will help understand their roles in normal physiology and/or pathological states associated with diseases. A successful approach we are using to try to fill this gap is genetically encoded markers for proteins to follow dynamics in light microscope systems, then high-resolution imaging by electron microscopy. We can count on an expanding molecular probes toolbox to assess protein location and function, considering, for every target protein, the appropriate tag and methodology. For example, while most proteins tolerate tags, some proteins require specific or smaller tags resistant to proteases or different subcellular microenvironments. Importantly, the probes reviewed here (Fig. 1) are appropriate to the capabilities of sophisticated multiscale imaging tools that can be used in a complementary manner to examine the entirety of events associated with complex biological questions. The ultimate goal is to visualize macromolecules of interest with high spatial and temporal resolution, potentially generating the 4D maps of their distribution and interactions within a given tissue or organism.

Common challenges

EM-level mapping of the distribution of specific proteins in situ has proven vital to the understanding of many cellular functions, but most of the methods previously developed have fallen short of the resolution afforded by electron microscopy and suffer from a range of seemingly intractable limitations. These limitations include procedures that do not adequately preserve cellular and supramolecular ultrastructure and native protein distribution, lack the capability to deliver uniform 3D labeling, and generally provide suboptimal resolution of the contrasting agent. All of these limitations greatly limit the ability to interpret the imaging data. Compounding this is the fact that many of these approaches involve complex procedures and specialized instrumentation that made their use challenging technically. For example, antibody-based protein detection, the most widely used approach, usually requires a compromise in chemical fixation to maintain antibody-epitope binding and the use of permeabilizing detergents to facilitate access of the labeling reagents, both of which significantly perturb cellular ultrastructure. The introduction and refinements of molecular-genetic approaches for EM-level protein localization allowed incorporation of tags and labels directly into the target protein and offered the ability to overcome many of the aforementioned limitations, in addition to facilitating excellent correlated light and EM. Below, we review the probes currently available to the research community and examples of their application in addressing biomedical challenges.

Tetracysteine tags

Fluorescent labeling of tetracysteine-tagged (4Cys) proteins followed by photooxidation of diaminobenzidine (DAB) was one of the first examples of a genetically encoded light microscopy (LM)/EM-compatible approach [9] and has been very successful in a host of diverse applications. Tetracysteine tags are short encodable peptides having the general sequence Cys-Cys-Xaa-Xaa-Cys-Cys and can be fused to either the amino or carboxyl terminus of the protein of interest or can be incorporated into internal sites. Tetracysteine tags exhibit sub-picomolar affinity for a variety of membrane-permeable biarsenicals such as the green fluorescent *Fl*uorescein *Ars*enic *H*airpin-binder (FlAsH) (ex 508, 528 em) and the red fluorescent Resorufin-based AsH (ReAsH) (ex 593, 608 em), and the latter is capable of photooxidizing DAB to create an EM-visible reaction product. Exhaustive screening of a clone library of internal and flanking sequences around the tetracysteine motif in living mammalian cells revealed the consensus sequence FLNCCPGCCMP exhibits the highest affinity, stability, and quantum efficiency of the 4Cys-biarsenical complex thus far. One powerful application of the 4Cys labeling methodology is to temporally and spatially distinguish new vs. old proteins using spectrally distinct fluorescent biarsenical ligands that only fluoresce when bound to the genetically appended tetracysteine protein tag. We refer to this as in vivo *optical pulse-chase labeling*. Numerous researchers employed the small 4Cys tag as it is especially effective in situations that require minimally perturbing modifications to proteins such as labeling of small viral proteins [22]—or situations that require live-cell imaging of in vitro preparations by light microscopy [15]. Use of these 4Cys constructs to follow the dynamics of beta-actin is a good example of the usefulness of a small

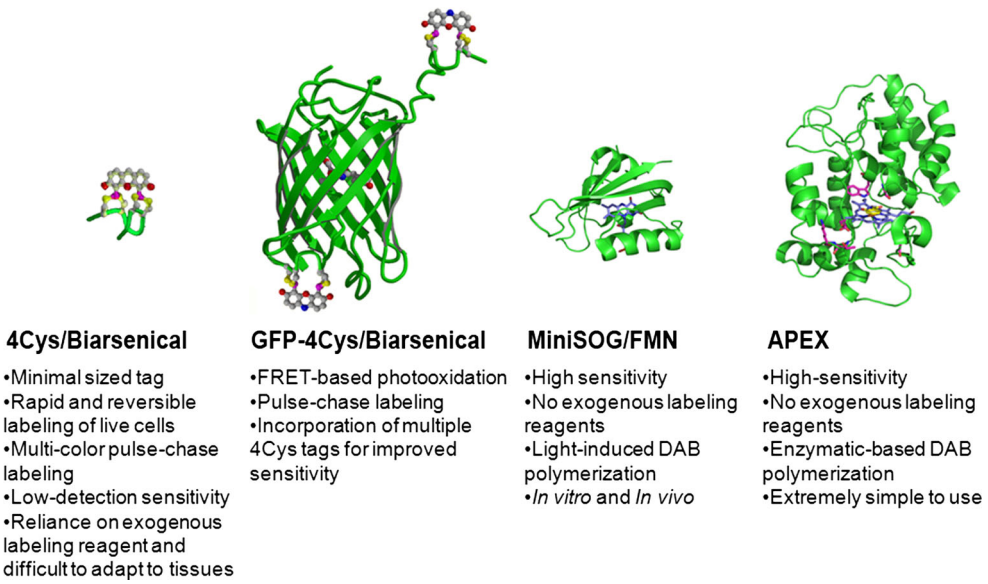

Fig. 1 Comparison of genetic probes for CLEM. Diagram of different tagging systems described in this article and important facts regarding their application

probe [8]. With this system, it is possible to label the 4Cys-tagged actin with FlAsH with high specificity for live-cell observations, and to subsequently fix the cells and then counterstain to mark other structures with a variety of other labeling methods (Fig. 2). Living cells labeled with ReAsH can also be used for time-lapse imaging followed by glutaraldehyde fixation to arrest cellular activity, and then ReAsH can be used to photooxidize DAB for subsequent highly correlated EM (Fig. 3). Another powerful application is to incorporate 4Cys motifs into fluorescent proteins such as green fluorescent protein (GFP) in order to allow them to be used for both light and electron microscopy [10]. Labeling of GFP-4Cys with ReAsH allows a DAB reaction product to be generated either by direct excitation of the ReAsH or by exciting the ReAsH by Förster resonance energy transfer (FRET) from the GFP (Fig. 4). The main drawbacks to 4Cys tags are that (1) they require the addition of the exogenous biarsenical-labeling reagent ReAsH to enable DAB photooxidation and (2) control of background staining due to the lipophilic nature of this biarsenical requires careful work to determine labeling and washout conditions [12]. Furthermore, our effort to adapt this labeling technology to complex tissues and whole organisms has proven difficult due to the requisite stringent labeling conditions and disruptive oxidative effects of aldehyde fixation on the tetracysteine motif [12]. Finally, due to the modest reactive oxygen produced by ReAsH, it is not the tool of choice today for imaging low-abundance proteins by EM, when compared to *mini singlet oxygen generator* (MiniSOG) and *enhanced ascorbate peroxidase* (APEX), described in the following paragraphs.

MiniSOG

Since its introduction [20], MiniSOG, the first fluorescent protein genetically engineered specifically for correlated light and electron microscopy (CLEM), overcame the aforementioned limitations by having the intrinsic ability to efficiently photooxidize DAB, thereby providing EM contrast without

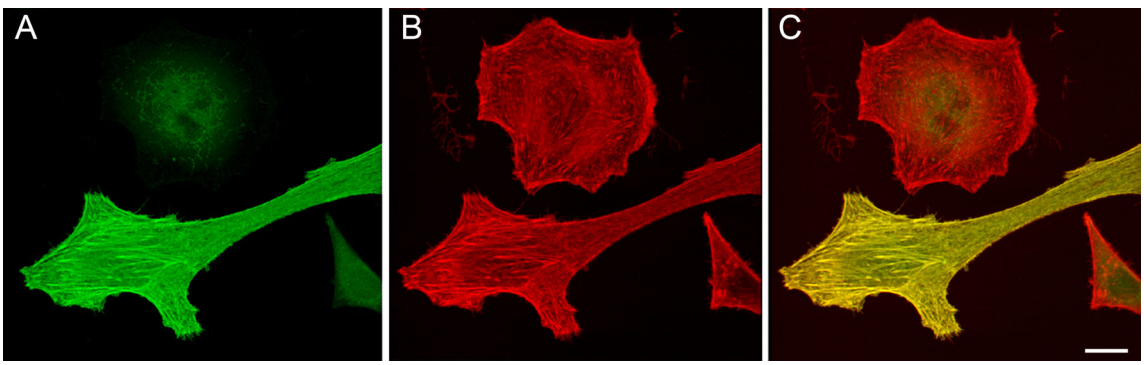

Fig. 2 Imaging of actin dynamics using the 4Cys technology. **a** Live-cell confocal imaging of tetracysteine-tagged beta-actin in cultured cells using the green fluorescent biarsenical FlAsH. The *lower cell* is expressing the recombinant protein while the *upper cell* is not. **b**, **c** Following fixation, cells counterstained with phalloidin-rhodamine show excellent co-staining in the transfected cell (*yellow*). *Residual green fluorescence* in the non-transfected cell is typical of background non-specific biarsenical cellular labeling (**a**). *Bar*=5 μm

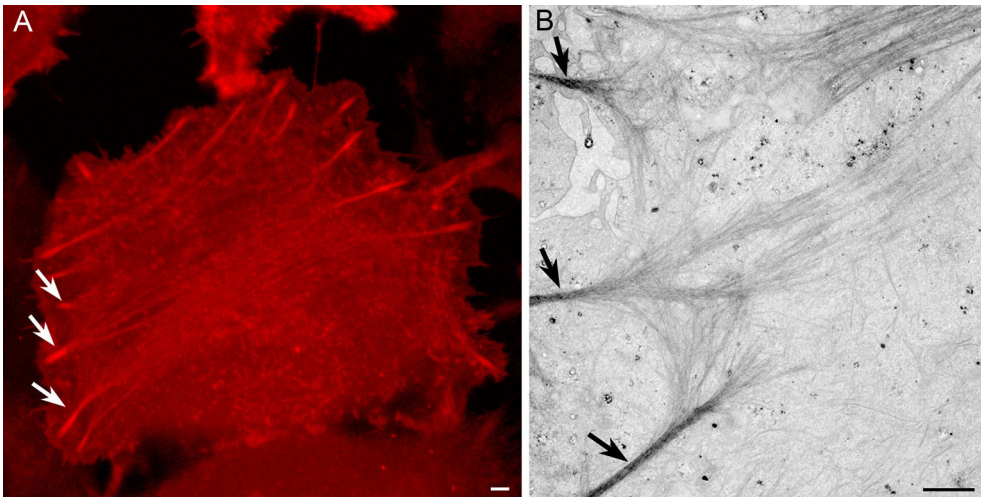

Fig. 3 CLEM imaging of cytoskeleton arrangement. **a** Following live-cell time-lapse multiphoton imaging of cells expressing tetracysteine-tagged beta-actin labeled with ReAsH, the cells are fixed and the ReAsH is used to photooxidize DAB into an electron-dense polymer visible by EM (**b**). *Arrows* indicate the same bundles of actin visible by both LM and EM. *Bar*=1 μm

the need for an extraneously applied labeling agent. Photooxidation using MiniSOG fusion proteins requires only the small molecules of DAB and dissolved molecular oxygen, which can readily penetrate fixed cells and tissues. This technique has been employed with great success in a wide variety of research activities [1–3, 6, 17, 19]. For example, it was used to visualize, for the first time, the dynamic organization of the small p53-silencing adenoviral protein E4-ORF3 in intact and well-preserved nuclei (Fig. 5; [19]).

TimeSTAMP-MiniSOG

MiniSOG has also been combined recently with the *time-specific tag for the age measurement of proteins* (TimeSTAMP) [16] and a split-fluorescent protein, creating the first fully genetically encoded, time-resolvable protein tag for correlated light and EM [5]. The use of this new type of molecular "pulse-chase" tool allows tracking of specific subpopulations of a target protein with high spatial and temporal resolutions. Specifically, this tool provides the ability to screen samples during a live imaging study using light microscopy as labeled copies of a target protein are being positioned in their cellular subdomains, and to visualize them both by fluorescence and at high resolution by EM. Applying the TimeSTAMP-specific protease inhibitor (BILN-2061) for a short incubation time (1–3 h) allows for the marking of new proteins made during the labeling pulse. In this way, one can visualize in living neurons newly synthesized tagged proteins in time-lapse LM imaging, and follow them as they trafficked through the expressing cell. EM analysis, including 3D electron tomography of labeled proteins, can reveal their localization at high resolution. Using

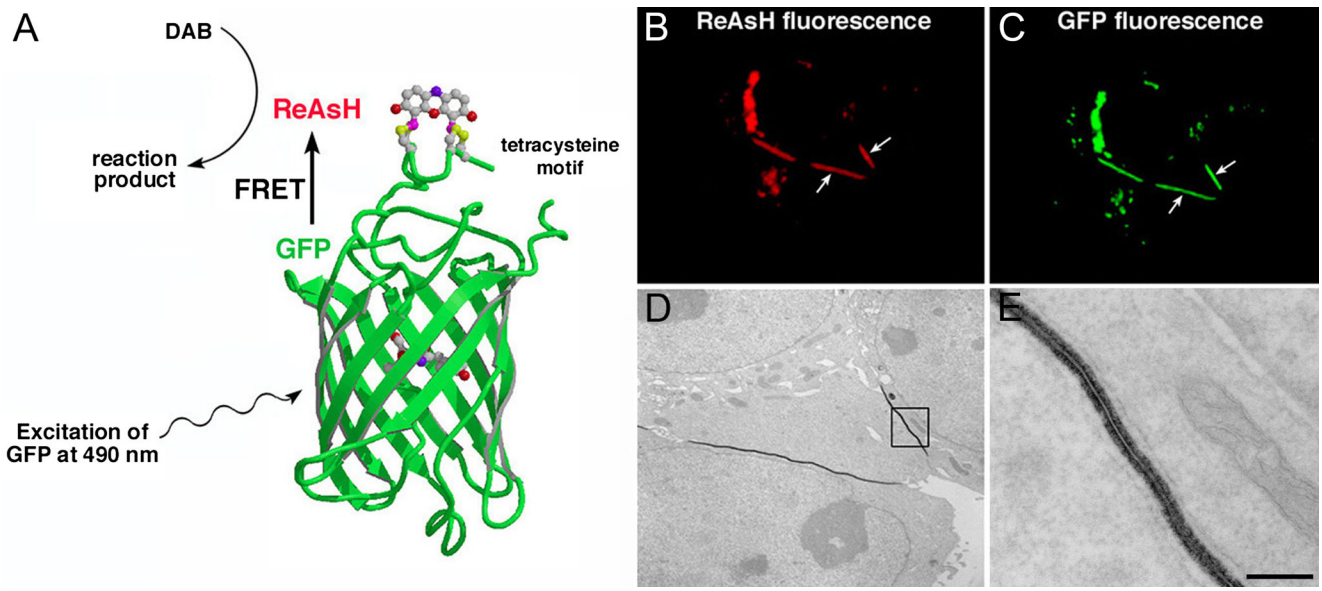

Fig. 4 FRET-driven photooxidation. **a** Small tetracysteine tags can be incorporated into fluorescent proteins such as GFP to enable direct or FRET-based photooxidation to enable EM visualization. **b**–**e** CLEM of cells expressing connexin43-GFP-4Cys labeled with ReAsH. *Bar*=200 nm

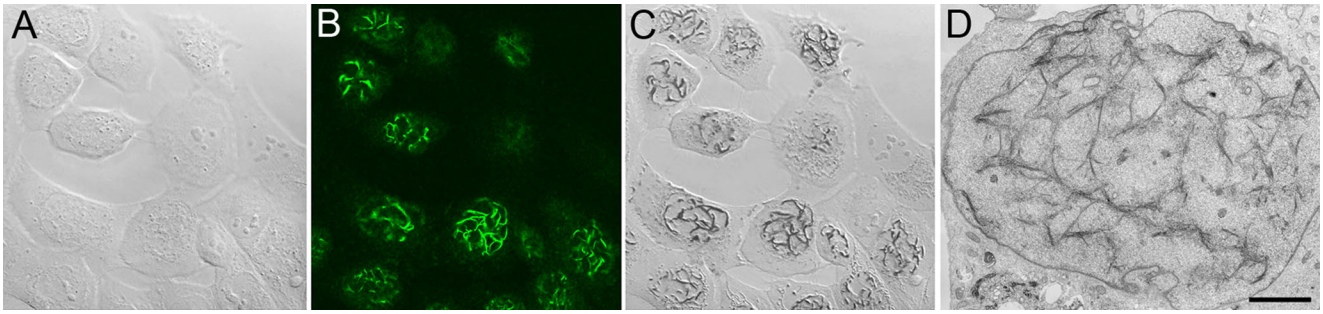

Fig. 5 CLEM imaging of cultured cells expressing the adenoviral protein E4-ORF3 fused to MiniSOG. **a** Transmitted light image prior to fluorescence photooxidation. **b** Confocal image of MiniSOG fluorescence showing an intranuclear filamentous network formed by E4ORF3. **c** Transmitted light image following fluorescence photooxidation showing DAB labeling corresponding to the fluorescence. **d** Electron microscopy of a reacted cell showing staining of the otherwise refractory elaborate filamentous network formed by E4ORF3. *Bar*=2 μm

TimeSTAMP for pulse-chase labeling has revealed previously uncharacterized aspects of the life cycle of the synaptic protein PSD95 [5]. Similarly, the application of this tagging system allowed the visualization of "longer lived" alpha-synuclein proteins as they traffic and accumulate at presynaptic terminals (Fig. 6).

APEX

APEX (for enhanced *A*scorbate *PE*ro*X*idase) [18] is a complementary probe utilized for protein labeling at the EM level. It is a small (40 % smaller than horseradish peroxidase, HRP), genetically encodable peroxidase engineered to express as a monomer with a high degree of enzymatic activity towards DAB. It also has the remarkable property, unlike HRP, of exhibiting high enzymatic activity in all cellular compartments, including cytoplasm, and does so after strong aldehyde fixation. While APEX is not fluorescent, it is very attractive as a probe because of its sheer simplicity and ease of use for labeling of proteins and making them visible to both light and electron microscopy. The protocol for labeling APEX-expressing fusion proteins in cells and tissues for EM is just one additional step in an otherwise conventional protocol to optimally preserve ultrastructure using glutaraldehyde primary fixation and osmium tetroxide post-fixation. In cells or tissues expressing an APEX-fusion protein, following glutaraldehyde fixation, the sample is incubated in a DAB solution containing 0.03 % hydrogen peroxide for as short as a few seconds to minutes. The formation of the reaction product can be monitored readily by ordinary transmitted light microscopy and the labeling intensity easily controlled. Since the reaction takes place in highly cross-linked glutaraldehyde-fixed specimens at 4 °C, diffusion of the reaction product can be minimized. Furthermore, since the labeling can be observed by ordinary light microscopy, many specimens can be screened rapidly prior to more time-consuming preparation for electron microscopy (Fig. 7). APEX has been used successfully to

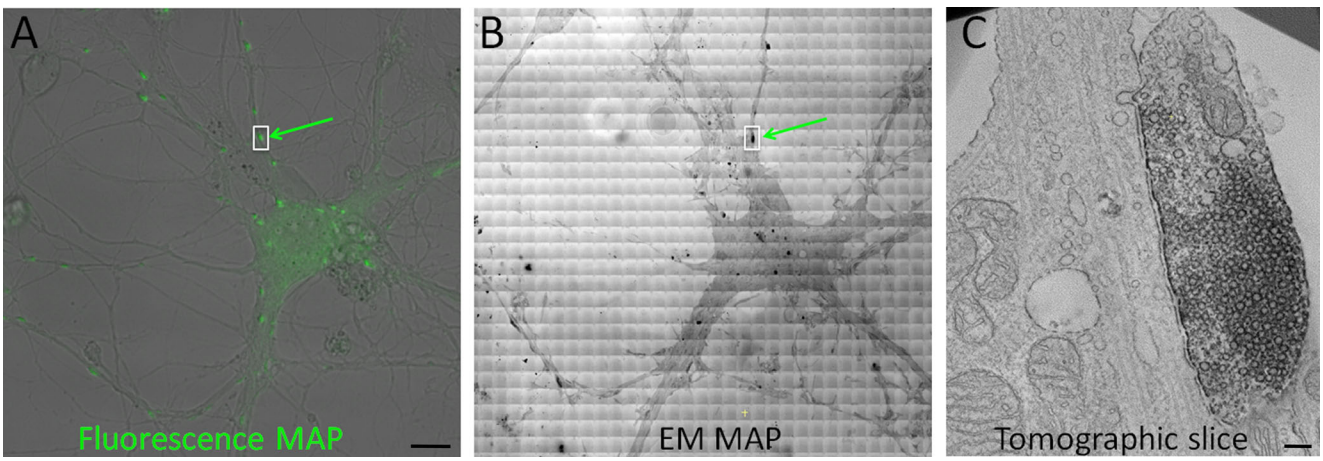

Fig. 6 Distribution of "older" AS-TimeSTAMP-YFP-MiniSOG proteins at presynaptic terminals in neurons by CLEM. YFP fluorescence map allows the visualization of labeled proteins. **a** Fluorescence image superimposed to transmitted light image. *Bar*=10 μm. Following photooxidation and EM processing, a correlated EM map (**b**) is used to identify target labeled areas (*green arrow*) to perform 3D electron tomographic analysis. **c** Representative slice of the electron tomogram corresponding to the presynaptic terminal indicated by the *green arrow* in **a**, **b**. The darker intensity of the signal reflects the AS labeling associated with various presynaptic endomembrane systems. *Bar*= 200 nm

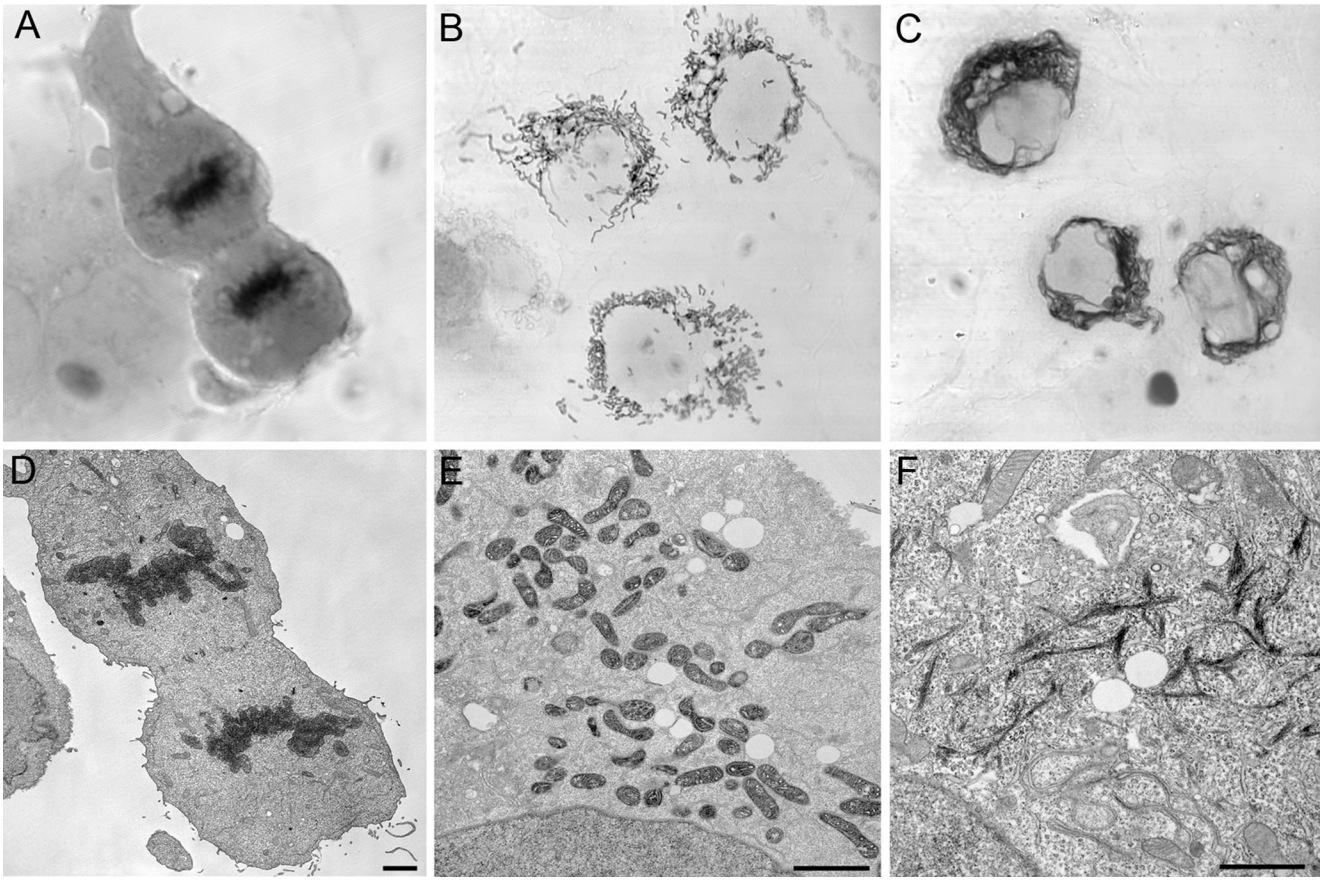

Fig. 7 Correlated light (**a**–**c**) and EM (**d**–**f**) imaging of a variety of APEX fusions in cultured cells. **a** Histone 2B-APEX in a dividing cell showing chromosome labeling. **b** Mitochondria matrix targeted APEX. **c** Cytoplasmic staining of vimentin-APEX. *Scale bars*=1 μm

localize a large number of cellular proteins, including actin, tubulin, vimentin, histone 2B, connexin43, PSD-95, and various mitochondrial proteins, in a wide variety of cell types with excellent results. Like MiniSOG, the ultrastructural preservation is excellent since no permeabilizing detergents or compromises to chemical fixation are required for labeling. However, in our work with APEX, we observed a limitation in its detection sensitivity, as some low-level expressing proteins were not always detectable. We hypothesized that the limited sensitivity of APEX may result from suboptimal folding or stability, poor heme binding, or some combination of these factors. These shortcomings helped to motivate an effort to identify key residues that could be modified in order to further improve these properties. This work led to the development of APEX2 via yeast-display-based directed evolution. APEX2 is a soybean-based ascorbate peroxidase that contains the original three APEX mutations (K14D, W41F, and E112K) plus an additional key modification (A134P) [14]. The APEX2 probe demonstrates significantly improved sensitivity when compared to the original APEX probe. This improvement allowed us to successfully localize a number of low-level expressing proteins including MICU1, a mitochondrial calcium uptake protein that was not possible to visualize using APEX. APEX probes can also be used for live-cell time-lapse imaging and CLEM when combined with GFP or other fluorescent proteins. We have successfully done this using a connexin43-GFP-APEX construct that, when expressed in living cells, exhibits the brightness, photostability, and low phototoxicity of GFP and, after chemical fixation and the addition of DAB and H_2O_2, is able to create a reaction product visible by EM (Fig. 8). Notably, we also observed a sensitivity of APEX to light; particularly intense blue light can deteriorate its enzymatic activity. To avoid this problem, fusions with yellow or red fluorescent proteins are recommended.

The APEX2 probe is likely to be adopted widely due to its ease of use and the fact that it overcomes so many obstacles encountered with other EM-level protein mapping approaches.

Tracking genetic labels across imaging modalities

A powerful application of these genetically encoded CLEM probes is to combine them with volume imaging by LM using confocal or multiphoton microscopy, followed by 3D EM imaging to enhance the ultrastructural reconstruction of specific cells and tissues. Since the labeling is done prior to the physical sectioning of the sample, these probes are perfectly suited to conventional serial section EM, electron

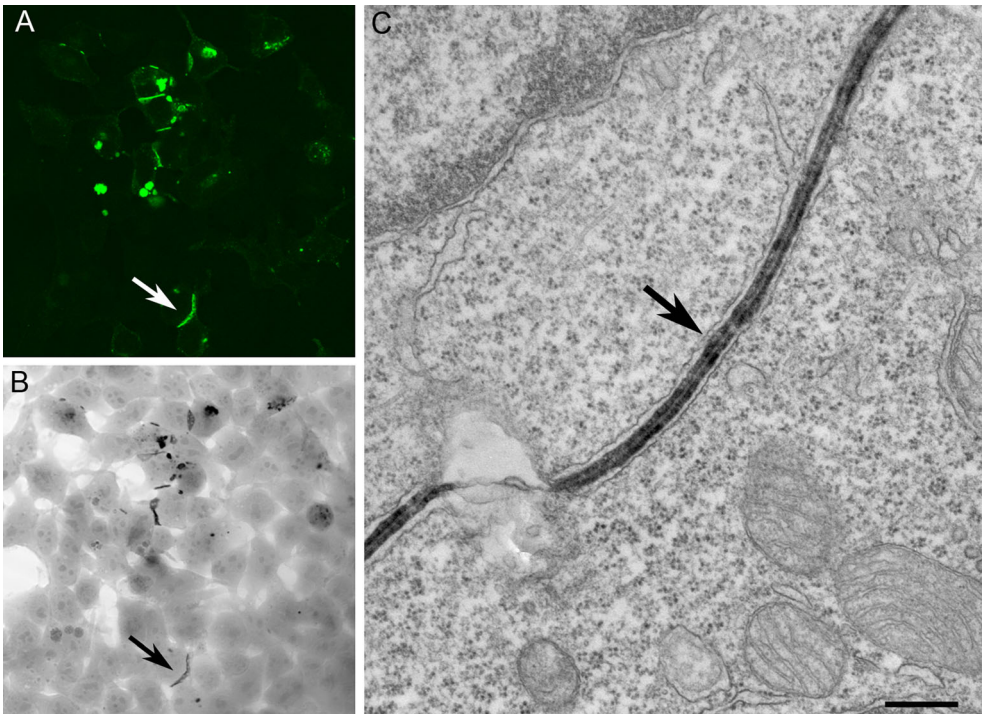

Fig. 8 CLEM imaging of connexin43-GFP-APEX. Labeling of the same gap junction (*arrows*) is visualized by **a** confocal, **b** transmitted light, and **c** electron microscopy. *Bar*=200 nm

tomography, and serial block-face scanning EM (SBEM). For example, we have used MiniSOG to enable the targeted labeling of retinal ganglion cell subtypes and subsequent 3D EM reconstruction. Mice expressing Cre recombinase in retinal ganglion cells were subjected to intravitreal injection with AAV virus carrying stop-floxed farnesylated MiniSOG. Following primary fixation with aldehydes, the retina was photooxidized (Fig. 9a) and then stained for SBEM imaging. However, the staining protocol for SBEM imaging involves intense labeling with heavy metals, which completely obscures the location of labeled ganglion cells in the specimen and renders the specimen completely opaque to light. Therefore, in order to track the cells of interest, X-ray microtomography was performed on the tissue to reveal the photooxidized cells in the stained tissue (Fig. 9b) [4]. The X-ray tomographic data allows for precise trimming and mounting of the specimen for serial block-face SBEM imaging. SBEM involves the iterative process of removing an ultrathin section of tissue from a specimen in the SEM, and then imaging the resulting block-face [7]. When this process is repeated

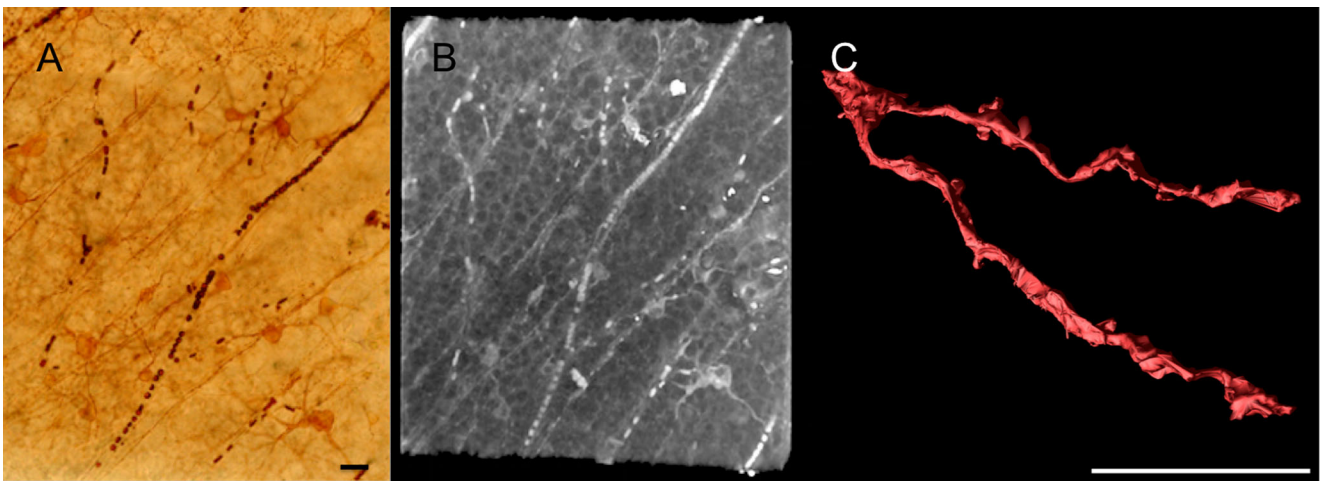

Fig. 9 Multimodal multiscale reconstruction of neurons using MiniSOG. The retinal ganglion cell subpopulation is specifically targeted for MiniSOG expression. **a** Cell bodies and neurites are clearly visible in transmitted LM following photooxidation of DAB with MiniSOG. **b** The same region of interest is tracked by X-ray microscopy following infiltration of the tissue with heavy metals. The DAB-labeled neurons and tissue landmarks such as blood vessels are visible in the X-ray tomogram. **c** Following SBEM imaging, 3D segmentation of dendrites from ganglion cells is facilitated by the presence of DAB. *Scale bars*=10 μm

hundreds or thousands of times, a large 3D volume is generated with nanometer resolution. The labeled cells are electron-dense due to DAB deposition and are consequently significantly easier to track and segment for large-scale cellular reconstruction (Fig. 9c).

Conclusions

The development of the next-generation molecular-genetic and chemical-labeling approaches will advance the application of high-resolution, correlated, multiscale, and multimodal microscopy to biomedical research. With these expanding methods, we will elevate our ability to determine the locations of specific proteins in situ, a capability that has proven important in biology where localization of molecular constituents in cell and tissue contexts has provided important new understanding. The EM-compatible probes presented here consist of complementary tools, each presenting different strengths as well as limitations, and are not meant to serve as a comprehensive list of those available for CLEM, but rather ones we helped to develop. Indeed, new molecular-genetic imaging probes and techniques are continually under development with new capabilities [13], and one can expect this trend to continue into the future. Ultimately, the end-user will need to match the tag that best fits the desired goal in the context of the specific biological question, taking into account the target protein and their subcellular environment, the spatial and/or temporal resolution desired, the size of the tag, and the approach utilized to make them visible by EM. Whether one chooses to combine the probes or to apply different ones in parallel, their application by CLEM allows visualizing in 3D a desired molecule in the context of extremely complex cell morphology and with respect to other key cellular structures, which is a prerogative of CLEM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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