FluoroNanogold: an important probe for correlative microscopy

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Abstract Correlative microscopy is a powerful imaging approach that refers to observing the same exact structures within a specimen by two or more imaging modalities. In biological samples, this typically means examining the same sub-cellular feature with different imaging methods. Correlative microscopy is not restricted to the domains of fluorescence microscopy and electron microscopy; however, currently, most correlative microscopy studies combine these two methods, and in this review, we will focus on the use of fluorescence and electron microscopy. Successful correlative fluorescence and electron microscopy requires probes, or reporter systems, from which useful information can be obtained with each of the imaging modalities employed. The bi-functional immunolabeling reagent, FluoroNanogold, is one such probe that provides robust signals in both fluorescence and electron microscopy. It consists of a gold cluster compound that is visualized by electron microscopy and a covalently attached fluorophore that is visualized by fluorescence microscopy. FluoroNanogold has been an extremely useful labeling reagent in correlative microscopy studies. In this report, we present an overview of research using this unique probe.

Keywords FluoroNanogold · Nanogold · Correlative microscopy · Ultrathin cryosections · Immunocytochemistry

Introduction

In this review, the term correlative microscopy refers to examining the same exact structures within a biological specimen (e.g., a sub-cellular feature) using two, or more, imaging modalities. The goal of correlative microscopy is to provide greater information than can be provided by any individual imaging method. A number of biological questions have been approached using different aspects of correlative microscopy. However, combining fluorescence and electron microscopy remains the most common form of correlative microscopy. Different aspects of correlative fluorescence and electron microscopy have been discussed in a number of excellent reviews and articles; the reader is referred to these for a broader discussion of correlative microscopy (e.g., [5, 10, 24, 40, 42, 58, 67, 68, 70, 83, 84, 92, 109]). Herein, we will focus on correlative fluorescence and electron microscopy using the bifunctional probe, FluoroNanogold (FNG). Additionally, we will discuss the merits of using ultrathin cryosections, particularly of tissues, as the labeling substrate for correlative microscopy.

Several fluorescent markers have been used in correlative microscopy or, though untested, have the potential to be useful. These include the various genetically tractable fluorescent proteins, a large number of small fluorescent molecules, quantum dots, and fluorescently labeled gold nanoparticle conjugates (FNG). However, to be useful for correlative fluorescence and electron microscopy, the fluorescent probe must also be detectable by electron microscopy. That is, it must be inherently electron dense, like quantum dots, or gold

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nanoparticles (AuNPs). Fluorescent probes themselves lack electron density and cannot be directly rendered electron dense; however, some of these can be detected by electron microscopy using an indirect photooxidation method (see below).

Quantum dots are nanometer-sized crystalline particles that are fluorescent and possess some electron density and thus can be detected by both fluorescence and electron microscopy. An interesting feature of quantum dots is their size is correlated with the color of fluorescence emitted [6]. Another important feature of affinity labels is the ability to couple affinity reagents (e.g., antibodies) to them. Several different biomolecules have been conjugated to quantum dots [2]. However, problems with quantum dots, including stability, blinking, and low electron density, have limited their use as correlative probes. The use of quantum dots for labeling in biological systems has been reviewed and will not be discussed further ([16, 25, 26, 41, 50, 73]).

Attempts to prepare combined fluorescent and gold labeled probes by the adsorption of fluorescently labeled antibodies to colloidal gold particles have produced limited success [12, 13, 27], due primarily to quenching of the fluorescence by the gold particle absorption [45]. Gold nanoparticles efficiently absorb fluorescence, and this becomes a severe problem in larger gold particles. Generally, ≥ 10 -nm gold particles are unusable. In one study, a dual-labeled probe prepared by adsorption of fluorescein isothiocyanate labeled protein A to 20-nm colloidal gold particles produced fluorescent staining similar to that found with FITC-protein A without colloidal gold [90]; however, given the known tendency of colloidal gold conjugates to dissociate in solution [51], the fluorescence staining may arise from the binding of dissociated fluorescently labeled protein A. A simple test of ≥ 5 -nm AuNP-fluorescent conjugates to ensure a dual label is to centrifuge the probe: The dense AuNPs will pellet, and if all of the fluorescence is in the supernatant, that would indicate dissociation and/or nearly complete quenching. In addition, 20-nm colloidal gold conjugates are large and may be unable to penetrate or access sterically hindered binding sites, which could still then be labeled with even small amounts of the much smaller, unconjugated FITC-protein A. Penetration of particulate probes such as quantum dots and colloidal gold (CG) into cells or tissues is an important consideration for efficient labeling. Modest penetration of quantum dots into fixed cells that were permeabilized has been reported [14]. Colloidal gold particles have only very limited ability to penetrate into cells and tissues and certain organelles (see below).

There are numerous fluorescent probes that are not and cannot be rendered electron dense directly. Fortunately, a number of these fluorescent molecules can be detected at the electron microscope level. This is through the use of 3,3'-diaminobenzidine tetrahydrochloride (DAB), which was introduced as a detection system for electron microscopy for

the localization of peroxidatic activity [29]. The DAB precipitates at the sites of peroxidatic activity as a brown reaction product. Following exposure to osmium tetroxide, the DAB reaction product is converted to an electron dense product known as osmium black that is readily detectable by transmission electron microscopy (TEM).

When certain fluorophores and fluorescent polypeptides are excited with light of the appropriate wavelength under well-oxygenated conditions, they emit fluorescence and produce reactive oxygen species. It has been shown that excitation of certain small fluorophores as well as fluorescent polypeptides can also lead to precipitation of DAB under appropriate conditions ([11, 15, 28, 39, 55, 59, 60, 63, 97]). Coupling fluorescence microscopy with photooxidation of DAB followed by treatment of the sample with osmium tetroxide is a powerful tool for correlative microscopy (e.g., [20]). A more recent extension of this approach is the catalytic deposition of silver (termed "EnzMet") by peroxidase, leading to a more electron dense deposit than DAB ([64, 77]).

While this report deals with correlative fluorescence microscopy and thin-section electron microscopy, it should be noted that there are other forms of correlative microscopy. Examples of correlative microscopy lacking a fluorescence component have provided insight into certain biological questions. Coupling live-cell phase contrast optics with thin-section TEM has been used in the analysis of mitosis in cultured cells [48, 81]. In these studies, features associated with mitosis were followed over time, and at selected points in the mitosis process, the cells were fixed and processed for electron microscopy so that the features observed with phase contrast microscopy could subsequently be correlated with the higher-resolution TEM images. Another example is found in the analysis of the behavior of fibrinogen receptors by monitoring CG coated with fibrinogen using video-enhanced differential interference contrast optical microscopy and correlating that data with high-resolution scanning electron microscopy (SEM) [71].

In another study, fluorescence microscopy was coupled with whole-mount TEM [54]. Macrophages were cultured on formvar-coated electron microscope grids and then incubated with a fluorescent marker of pinocytosis (Lucifer yellow). Live-cell images were collected and the cells were subsequently fixed. The fixed macrophages were processed for detection of activity of the enzyme acid phosphatase using a cerium-based enzyme cytochemical method [82]. The cells were then subjected to critical point drying so they could be observed as whole-mount preparations. The electron-dense cerium reaction product was readily detected by TEM. We found that the Lucifer yellow tracer reached the acid phosphatase-positive compartments.

The various forms of super-resolution fluorescence microscopy developed over the past few years allow us to delve more

deeply into sub-cellular structures with fluorescence microscopy than was possible in the past (e.g., [4, 32, 91, 110]). However, there are still limitations to this technology. Electron microscopy continues to have the ability to resolve structures smaller than those resolved by super-resolution fluorescence microscopy. An important additional consideration is that in fluorescence microscopy, it is only the fluorescence signals that are observed; the rest of the sample is invisible. Thus, what is missing in fluorescence microscopy is the context in which the fluorescence can be placed. The non-fluorescent structures within a cell or tissues have been referred to as the “reference space” [30]. Correlative microscopy, combining fluorescence and electron microscopy of the same exact structures, allows for a better understanding of the fluorescent signal within the context of the cell—the reference space.

Nanogold

Traditionally, in immunoelectron microscopy, the predominant reporter system has been colloidal gold (CG; [89]). The most commonly used CG for affinity labeling, including immunolabeling, has been the 5-, 10-, and 15-nm-sized particles. The different sized CG can be easily distinguished from each other by electron microscopy (EM); distinguishing CG by their size facilitates double and triple labeling of the same

sample. However, it has been noted in several studies that there is an inverse relationship between CG size and labeling intensity (e.g., [85, 99]). Additionally, ultrasmall CG probes (1–3 nm) have been introduced (3; Van de Plas and Leunissen, 1993). Gold cluster compounds, which are discrete chemical compounds and thus not colloidal in nature, have also been introduced [33, 34]. Commercial preparations of a 1.4 nm cluster compound, known as Nanogold (NG), are now available. One advantage of NG compared to CG is that a variety of molecules can be covalently linked to NG [36], including antibody Fab' [34] or even ScFv fragments [80], peptides [96], oligonucleotides [79], lipids [35, 37], enzyme substrates [61], and toxins [43]—molecules that do not conjugate well to CG. On the other hand, molecules that adsorb to CG do so through less precise electrostatic or hydrophobic interactions.

Nanogold, the predecessor and a component of FNG, is a 1.4-nm gold cluster coordination compound that can be conjugated with various affinity probes including antibodies and streptavidin through discrete cross-linking reactions of incorporated reactive groups such as maleimides or *sulfo-N*-hydroxysuccinimides [1]. Under certain imaging conditions, such as high-magnification dark-field scanning transmission electron microscopy (STEM), NG can be visualized directly [34]. However, when used to label structures in cells or tissues, NG is often not readily visible with conventional imaging methods. NG can be rendered visible by autometallography

Fig. 1 Immunoelectron microscopic localization of caveolin-1 in cultured endothelial cells. **a** Conventional thin-section electron micrograph of a portion of a cultured human umbilical vein endothelial cell (HUVEC) illustrating the morphological appearance of caveolae (arrows). This cell was sectioned parallel to the substratum on which the cell was attached. **b** Immunoelectron microscopy detection of caveolin-1 in caveolae using NG as the reporter system in a preembedding localization procedure as we have described [88]. Caveolae were heavily decorated with silver-enhanced NG particles (arrows) while other structures such as coated vesicles were not labeled (arrowheads). This cell was also sectioned

parallel to the substratum. The anti-caveolin-1 antibody was characterized previously [57]. **c** Control incubation in which the primary antibody was omitted but all other incubation steps were the same as used on panel B. Note that the caveolae were free of silver-enhanced NG (arrows). **d** The sample was processed in the same manner as in panel B except that the section was cut perpendicular to the substratum on which the cell grew. The classical omega shape of caveolae can be better seen in this type of section. The heavily decorated caveolae are indicated (arrows). Bars= 100 nm

procedures such as silver or gold enhancement reactions (e.g., [35, 37, 99]). A very useful attribute of NG is that it penetrates further into cells or tissues and certain organelles than colloidal gold probes [99]. An example of this relates to labeling tubulin within centrioles where it has been difficult to achieve

significant labeling with CG (e.g., [21]). This was thought to be due to the poor penetration of the CG probes into this organelle. On the other hand, a high level of centriolar labeling was achieved when antibodies to tubulin were detected with NG and silver enhancement [85]. Another useful feature of NG is

Fig. 2 The comparison of the axial resolution in sections of human placental terminal villi at various section thicknesses is illustrated. A cryostat section (5 μm thick) was immunolabeled for the fluorescence detection of caveolin-1 α (CAV-1 α) (red) and the endothelial marker protein CD31 (green); the nuclei were detected with DAPI (blue). The preparation was then observed with a conventional microscope (a–d). Note that there was an overlap of the fluorescence signals that was most evident when all three colors were displayed simultaneously (c). The same exact structures were also observed with a confocal microscope (e–h). The blurring of the image due to out-of-focus signal is greatly improved with the confocal microscope (g). Another placental sample was processed to produce ultrathin cryosections (100 nm thick) and then labeled for detection of CAV-1 α , CD31, and nuclei in the same manner as with the 5- μm section (i–l). There was an apparent

improvement in resolution with ultrathin sections when compared to the optical section obtained with the confocal microscope. The CAV-1 α labeling was more readily detected as individual dots (representing individual caveolae) (compare (e) and (i)). The CD31 labeling was generally resolved as two layers in ultrathin cryosection (the more heavily labeled luminal plasma membrane and the abluminal plasma membrane with less label) (compare (f) and (j)). In all panels, the lumens of the capillaries are indicated (asterisk) as are the syncytiotrophoblast layers (arrowheads). In panels (i)–(l), the endothelium is indicated (arrow) as is the pericyte (double arrows). The DIC images (d, h, l) provide the morphological context in which the fluorescence signals can be placed. This figure is reproduced with the permission of the Journal of Electron Microscopy. Bars=10 μm ; inset bar=500 nm

that it labels to a higher density than colloidal gold [86, 100], due to its small size and the fact that it may be conjugated readily to antibody Fab' fragments rather than whole IgG molecules and does not require additional macromolecules (such as bovine serum albumen) for stabilization. The high labeling density achieved when using NG, coupled with silver enhancement, as the detection system is illustrated by the localization of caveolin-1 in cultured endothelial cells using a pre-embedding labeling procedure (Fig. 1). In this case, the plasma membrane microdomains known as caveolae were heavily decorated with silver-enhanced NG particles.

Silver-enhanced NG has been used as the electron microscopic label for correlative confocal and electron microscopic studies of insect neurons. These were labeled intracellularly with neurobiotin or biocytin, sectioned, and processed with both NG-Streptavidin for electron microscopic observation,

and streptavidin-Cy3 for confocal microscopic study and then embedded in epon/araldite. Interesting areas of the labeled neuron were imaged in the epon/araldite blocks using laser scanning confocal microscopy and then thin-sectioned at the indicated depth for electron microscopic study [98].

The utility of ultrathin cryosections for multicolor fluorescence microscopy and correlative microscopy

Ultrathin sections have been used in immunoelectron microscopy for many years. These sections have been derived from resin-embedded specimens (e.g., [90]) and from sucrose-embedded frozen sections [106]. The latter are generally referred to as ultrathin cryosections. Both types of sections have

Fig. 3 A model illustrating the advantage of using ultrathin cryosections for higher-resolution immunofluorescence microscopy. The diagram represents a placental capillary immunolabeled for CAV-1 α (red) and CD31 (green) and cut into sections of different thicknesses (5- μ m cryostat section, 500–750-nm optical section from the 5- μ m cryostat section, and 100-nm ultrathin cryosection). The luminal (LS) and abluminal (AS) surfaces are shown in green for CD31 while individual caveolae are shown in red. In the full thickness 5- μ m cryostat section, the green and red signals are stacked within the volume of the section giving the impression of co-localization (yellow). Elimination of much of this false co-

localization is obtained with the confocal optical section. However, it is difficult to resolve the individual caveolae, and there remains some false co-localization. The thinner ultrathin cryosection (100 nm) allows for more precise detection of individual caveolae; there is a low probability that individual caveolae will be stacked on top of each other since the caveolae (~70 nm in diameter) just fit within the physical section. Furthermore, the likelihood of overlap between the CD31 and CAV-1 α signals is further minimized in the ultrathin cryosection. This figure is reproduced with the permission of the Journal of Electron Microscopy

been successfully used for immunolabeling with CG as the reporter system [8, 22, 31, 47].

Cultured cells are amenable to preembedding labeling methods for immunoelectron microscopy (see Fig. 1). Solid tissue samples are less amenable to preembedding methods for a number of reasons, including poor penetration of reagents into tissues. For this reason, among others, tissues are generally sliced into sections of various thicknesses, including ultrathin sections (50–100 nm), prior to affinity labeling procedures such as immunolabeling. We have shown that ultrathin cryosections offer several advantages for immunolabeling [69, 104].

Ultrathin cryosections (100 nm) have been used for multi-color fluorescence microscopy and, when compared to confocal microscopy optical sectioning of standard-thickness cryosections (e.g., 5 μm), display higher-resolution images [69]. Immunofluorescence localization of caveolin-1 and CD31 (an endothelial marker-protein) in human placenta illustrates the point that ultrathin cryosections provide higher-resolution images than found in optical sections by confocal microscopy (Fig. 2). In capillary endothelial cells in the human placenta, and in other tissues as well, the luminal plasma membrane and the opposite abluminal plasma membrane can be very close together, separated by distances of only 100–200 nm in many instances. It is generally easy to distinguish these luminal and abluminal plasma membranes in ultrathin cryosections, but more often, it is difficult to do so in confocal optical sections of thicker specimens (compare Fig. 2e, i, f, j). We have modeled this improvement in resolution for the case of

localization of CD31 and caveolin-1 in human placental capillary endothelium (Fig. 3). (Human cells and tissue presented in this review were obtained following informed consent with protocols approved by the Biomedical Sciences Institutional Review Board at Ohio State University or the Nippon Medical School Hospital Ethics Committee.)

The improved resolution for fluorescence signals within ultrathin cryosections when compared to optical sections with confocal microscopy is due to improvements in the axial resolution. There is essentially no out-of-focus signal that can degrade the image with ultrathin sections. With ultrathin cryosections, all of the fluorescence must be derived from the physical section (50–100 nm in thickness); however, with optical sections derived from confocal microscopy, the axial resolution typically ranges from 500 to 700 nm depending on the objective lenses employed. Thus, there can be a 10-fold improvement in the axial resolution with ultrathin cryosections. While the x - y resolution obtained with ultrathin cryosections using conventional wide-field or confocal microscopes remains at the diffraction limit (200 nm), the improved axial resolution can lead to the appearance of improved resolution in the x - y dimensions. Additionally, Micheva and colleagues [66] point out that x - y resolution can be affected by spherical aberration that can degrade images. In confocal microscopy where the sample may be 5–10 μm thick, for example, they have estimated that degradation of lateral resolution may exceed a factor of two due to spherical aberration. Observing ultrathin sections with conventional high numerical aperture (NA) objective lens minimizes the problem of

Fig. 4 False co-localization of fluorescence is minimized in ultrathin cryosections. An ultrathin cryosection of human placenta has been labeled for the detection of CAV-1 α (green) and early endosome antigen 1 (EEA1) (red). Nuclei were labeled with DAPI (blue). The CAV-1 α signal was detected in capillary endothelial cells and pericytes (P) but not in the syncytiotrophoblast layer (arrowheads). The lumen of the capillary is indicated (star). The EEA1 signal is most robust in the syncytiotrophoblast layer (arrowheads) with lesser amounts in the

endothelium and pericytes (arrows). The DIC image with DAPI labeling is provided to give the morphological context in which to place the fluorescence signals. The borders of the capillary and the pericytes are indicated with the white lines. In the merged image, the EEA1 signal was distinct from the CAV-1 α signal even though the structures were in very close proximity. This figure was reproduced with permission of Methods in Molecular Medicine

spherical aberration thus leading to high-quality fluorescence images. Another feature of using ultrathin sections for fluorescence microscopy is that the section thickness is below the depth of focus of high NA objective lens further improving the axial resolution [19] This was evident in Fig. 2 where the closely spaced luminal and abluminal plasma membranes of capillary endothelial cells were readily separated from each other in the ultrathin cryosections. In contrast, these closely spaced plasma membranes were often not detected as separate structures in the confocal images.

We have also suggested that false co-localization in multi-color fluorescence microscopy is less likely to occur in ultrathin cryosections than in confocal optical sections of thicker specimens. This is due to the extreme thinness of the ultrathin cryosections, where it is less likely that separate fluorescent signals will be stacked on top of each in the physical ultrathin section than it is in a confocal optical section. This is illustrated by the dual localization of caveolin-1 α (CAV-1 α) and early endosome antigen 1 (EEA1) in the human placenta (Fig. 4).

We have also modeled the proposition that false co-localization is less likely in ultrathin cryosections than in confocal optical sections of thicker specimens (Fig. 5).

FluoroNanogold and correlative microscopy

FluoroNanogold is a bi-functional labeling probe consisting of a 1.4-nm gold cluster compound (NG) and a fluorophore conjugated to an antibody Fab' fragment, IgG, or to streptavidin. The composition of FNG is illustrated diagrammatically (Fig. 6). The great utility of FNG is that it can be imaged by fluorescence microscopy and subsequently by electron microscopy following autometallography to enhance the size of the gold signal such that it can be observed by electron microscopy in the context of cells and tissues. The fact that FNG can be observed by both fluorescence and electron microscopy makes it well suited to be a reporter system for correlative microscopy.

Fig. 5 A model illustrating how use of ultrathin cryosections can minimize false co-localization in immunofluorescence experiments. An idealized terminal villus from the human placenta is shown on the left side. Endothelial cells (1), a pericyte (2), and the syncytiotrophoblast layer (3) are depicted. Two different sub-cellular structures are shown in red and green. The thickness of an ultrathin section is indicated with the gray bar through the villus. The red and green fluorescent structures are shown in a side view (z-axis) and top view (x-y dimensions). Overlap of the red and green signals are depicted in yellow. The fluorescence signals

of red and green structures may be stacked above or below each other leading to false co-localization in the volume of the 5- μ m section. The situation is improved in the confocal optical section (z-resolution 500–700 nm); however, it is still possible for small structures to be stacked within the optical section, leading to false co-localization. The physical ultrathin cryosection (100 nm) minimizes the potential for false co-localization since small structures (≥ 100 nm) would occupy the entire section

FluoroNanogold - Fab'

FluoroNanogold - Streptavidin

Fig. 6 Diagrammatic view of FluoroNanogold Fab' and streptavidin conjugates. The fluorescent and gold labels are attached separately using discrete covalent cross-linking reactions. The 1.4-nm Nanogold particle is conjugated to Fab' at a hinge thiol by using a maleimido derivative, and to the streptavidin using an amine-reactive *sulfo-N*-hydroxysuccinimide (NHS) derivative, while fluorescent labels are attached at amino-sites elsewhere in the molecule using amine-reactive fluorescent labeling reagents, typically NHS- or *Sulfo*-NHS derivatives. For Fab' conjugates, this approach attaches the larger gold label at a position where it cannot obstruct target binding. Separate attachment spaces the fluorescent and gold labels apart to minimize fluorescence quenching

The initial use of FNG for correlative microscopy was carried out by us for the localization of components of intracellular granules in phagocytic leukocytes (Fig. 7). The labeling was carried out on ultrathin cryosections of human neutrophils in suspension. This also marked the first time that the exact same structures were imaged by fluorescence and electron microscopy in the same ultrathin section [87, 102]. This work thus served as proof of principle for conducting correlative fluorescence and electron microscopy on the same thin sections. Previously, fluorescence microscopy was carried out on thicker cryosections (200–500 nm), and the electron

Fig. 7 Correlative localization of myeloperoxidase (MPO) in human neutrophils using the same ultrathin cryosections for both fluorescence and electron microscopy. **A** Immunofluorescence localization of MPO in an ultrathin cryosection of isolated white blood cells using FNG as the reporter system. A reference cell is indicated (*arrow*) also present is an eosinophil (*e*). Bars of the EM grid are also indicated (*asterisk*). **A'** Electron micrograph of the same cells shown in panel (**A**) following silver enhancement of the FNG. **B** The cell indicated with the *arrow* in panel (**A**) is shown at higher magnification. Five MPO-positive granules are indicated with *arrows* and *numerals*. **B'** Electron micrograph is of the same region shown in panel (**B**). The MPO-positive granules are indicated with *arrows* and *numerals*. The MPO-negative granules are indicated (*arrowheads*). The electron micrograph provides the reference space in which to place the fluorescence signals. The fluorescent dots shown in panel (**B**) and the FNG silver-enhanced structures seen in panel (**B'**) indicate that there is a one-to-one correspondence. Bars: **A'**=5 μm; **B'**=0.5 μm

microscopy was done on adjacent thin sections [23, 108]. While this approach can provide useful information, it is not equivalent to examining the same section and thus the same exact structures by both fluorescence and electron microscopy. Since our initial use of the same ultrathin cryosection for correlative fluorescence and electron microscopy [87, 102], this approach has been used by others with ultrathin cryosections (e.g., [72]) and for resin-embedded samples (e.g., [19, 65, 94]).

Next, FNG as a labeling reagent for correlative microscopy was applied in the context of tissue sections [100, 101]. Correlative microscopy with solid tissue samples is inherently more difficult than with cultured cells. However, we approached this problem using ultrathin cryosections using human placenta as the model tissue and FNG as the probe for visualizing the localization of several antigens. In these studies, ultrathin cryosections of placental tissue were collected on formvar-coated EN grids; the grids were “finder grids” containing a marker system so that the same exact locations

could be viewed by light and electron microscopy. One of the antigens localized for correlative microscopy was CAV-1 α , a marker for the membrane microdomains known as caveolae. Caveolae in placental endothelial are about 60 nm in diameter; thus, localization of CAV-1 α to these small caveolae represents a rigorous test for correlative microscopy. The fluorescence signals from FNG used to detect CAV-1 α were usually in small dot-like structures in placental endothelial cells. Following silver enhancement of FNG, examination of the same ultrathin cryosections by EM confirmed that individual dots observed by fluorescence microscopy were caveolae (Fig. 8). These data confirm that ultrathin cryosections of tissue coupled with the use of FNG as the reporter system represent an important method for correlative fluorescence and electron microscopy.

The bifunctional nature of FNG permitting both fluorescence and electron microscopy has been useful in other studies as well. For example, it has enabled the three-dimensional analysis of subcompartments in nuclei [9, 105]. FluoroNanogold has also been employed for correlative analysis of centromeres in plant chromosomes with fluorescence microscopy and SEM [93]. It has also been shown that ultrathin sections of Lowicryl K4M resin embedded tissues (retina and testis) can be used for

correlative fluorescence and electron microscopy with FNG as the labeling probe [19]. Thus, it is clear that FNG is a versatile labeling probe that can be applied in many biological settings and using a variety of methods.

It is important to point out that the correlative microscopy method developed by us (TT and JMR) using ultrathin cryosections for both fluorescence and electron microscopy [100, 102] was used to validate super-resolution PALM microscopy ([4]; see their Fig. 3). This further supports the idea that increased resolution is associated with ultrathin sections and fluorescence microscopy.

A diagrammatic summary of ways that fluorescence and electron microscopy of cryosections has been combined is given (Fig. 9). This includes correlative fluorescence and electron microscopy using the same ultrathin cryosection.

Future directions

The preparation of combined fluorescent and gold nanoparticle immunoprobe requires careful consideration of the possibility that the gold nanoparticles will quench the fluorescence of the fluorophore through resonance energy transfer,

Fig. 8 Detection of the localization of CAV-1 α with correlative immunofluorescence and immunoelectron microscopy in the same ultrathin cryosection of a tissue sample. **a** Fluorescence localization of CAV-1 α in a portion of a placental endothelial cell was typically in individual dot-like structures (arrows). In some cases, the fluorescence signals appeared fused (arrowhead). **b** The same structure shown in panel (a) was observed by EM following silver enhancement of FNG. The same exact sub-cellular features observed by fluorescence were detected by EM (arrow and arrowhead). A portion of the electron dense grid bar is evident (asterisk). **c** An enlargement of the area of panel (a) indicated by the

arrow is shown. Two dot-like structures are indicated (arrows and numerals). **d** An electron micrograph of the structures indicated in panel (c) is shown following silver enhancement of FNG. The same two structures initially seen with fluorescence are heavily decorated with silver enhanced particles (arrows and numerals). The omega shape of a typical caveolae is evident by EN (arrow #1). The nucleus (n) provides a portion of the reference space in which the fluorescence can be placed. Scale bar = 100 nm. The contrast of the ultrathin cryosection was enhanced with a positive-contrast method [103]

Fig. 9 Summary of methods employed for combining fluorescence and electron microscopy using frozen sections; the advantage of combining fluorescence and electron microscopy in the same ultrathin cryosection is also shown. **a** The classical approach uses thicker cryosections (0.2–2.0 μm) on coverslips for fluorescence microscopy and an adjacent ultrathin cryosection for electron microscopy. In this case, the same exact objects were not observed. **b** Ultrathin cryosections were mounted

on coverslips for fluorescence microscopy, and an adjacent ultrathin section was mounted on an EM grid for electron microscopy. Again, the same exact objects were not observed. **c** Alternatively, a single ultrathin cryosection was mounted on an electron microscope grid for both fluorescence and electron microscopy. In this case, the same exact objects were observed with both imaging methods

according to the mechanism elucidated by Förster [18, 111]. The magnitude of quenching is inversely proportional to the separation of the gold nanoparticle and fluorophore, but proportional to the degree of overlap (the “overlap integral”) between the emission spectrum of the fluorophore and the absorption spectrum of the gold. For the relatively small NG particle, the overlap is moderate, and the critical “Förster distance” at which 50 % quenching occurs ranges between about 4 and 7 nm. Thus, separate attachment of the NG to a hinge thiol, and the fluorescent labels to amino groups elsewhere in the Fab’, provides sufficient separation to allow bright fluorescence. However, for larger gold particles, the Förster distance increases significantly and conjugation of gold and fluorescent labels to the same probe results in drastic loss of fluorescence [45, 76]. For example, with 6-nm gold, useful fluorescence intensity requires that the gold and fluorophore be delivered to the target conjugated to separate antibodies [46], thus not achieving a true dual label probe.

Although the issue of fluorescence quenching has restricted the expansion of FNG technology to larger gold labels, a recent preliminary study using a covalently linked 5-nm gold and fluorescently labeled immunoprobe in which the fluorophore and gold separation were synthetically designed

to promote fluorescence enhancement through constructive interference provided evidence that combined fluorescent and gold labeling may be possible if other energetic interactions can compensate for Förster quenching [44]. As an alternative, we have also investigated the use of combined horseradish peroxidase and 1.4-, 5-, or 10-nm gold probes to deposit fluorescently labeled tyramide substrates, which are deposited within 10–100 nm of the enzyme and hence outside the Förster distance. Using this method, successful combined fluorescence and EM labeling was demonstrated using a 5-nm gold and Alexa Fluor 488 tyramide [78]. This method provides the additional benefit that it may also be used with chromogenic substrates, thus affording a method for correlative brightfield light microscopy, which reveals the morphological context, and EM.

The advent of super-resolution light microscopy has provided a revolutionary advance in the application of fluorescence labeling to structural microscopy, enabling a new degree of resolution. However, while it greatly expands the possibilities of fluorescence, it does not replace EM or correlative microscopy as a method for true molecular localization. The resolution achievable with super-resolution LM is insufficient to answer important biological questions that might hinge on

determining which side of a membrane a particular protein is located, or whether a feature of interest is a pre- or post-synaptic component. For example, super-resolution fluorescence microscopy revealed that the cytochrome C oxidase import sequence labeled with photoactivatable protein is localized within mitochondria in COS-7 cells; subsequently, EM revealed that the reporter molecules do not extend into the outer (~20 nm) mitochondrial membrane [4, 52]. This illustrates that correlative super-resolution fluorescence and electron microscopy [10, 26, 67] also yields more than either method alone and is more than just the “sum of its parts” [52].

Recent advances in preparative methods and instrumentation for cryoelectron microscopy and electron tomography [17, 38, 62], and the development of instruments such as the “Shuttle-and-Find” and iCorr correlative fluorescence and cryoEM systems that can track a region of interest between light and EM provide both opportunities and challenges for labeling technologies such as FNG. While probe administration and immunolabeling may be simultaneous, the processing required and the nature and dimensions of the specimen in which each signal is observed are different. The ability to observe both signals in the same specimen, and preserve both so that specimens may be transferred back and forth between observational modes and archived, will become increasingly important.

In this context, an important result has been successful correlative fluorescent and TEM imaging of transcription sites in the same specimen in 150-nm-thick ultrathin cryosections of fixed and pelleted cell cultures immunolabeled with FITC-protein A-colloidal gold [75]. Visualization of GFP expression by light and electron microscopy in LR White sections [56] and in Epon/araldite-embedded sections [98] demonstrates that fluorescence can be preserved in resin-embedded sections which may then be imaged by either fluorescence or EM methods. This has been confirmed by the correlative confocal and electron microscopy of rat chondrosarcoma cells expressing GFP/YFP fusion proteins embedded in LR white. Alternating 0.5 μ m semithin and 80-nm ultrathin sections were cut from the same block: confocal images from 0.5- μ m sections were overlaid atop TEM images of the same cells collected from the next serial ultrathin 80-nm section after a separate immunogold labeling procedure using a rabbit anti-GFP primary and 6-nm colloidal gold-anti-rabbit secondary [49]; similar procedures have been used for correlative fluorescence, brightfield light using peroxidase developed with DAB, and electron microscopy using successive resin-embedded sections [94, 95]. Recently, the use of uranyl acetate in acetone as a fixative, in the absence of glutaraldehyde, has been found to preserve fluorescence sufficiently for fluorescence microscopy even in ultrathin EM sections [74]; fluorescence from GFP variants may also be chemically reactivated in LR White, glycol methacrylate (GMA), or methyl methacrylate (MMA)-embedded specimens by treatment with 0.1 M sodium carbonate buffer, pH 11.6, for 2 min [112].

The development of serial section methods for electron microscopy, such as serial blockface SEM and focused ion beam (FIB) SEM, provides another opportunity for correlation between electron and light microscopic methods. While these methods do not currently achieve sufficient resolution to visualize individual NG particles, X-ray microscopy has been used as a guide to correlate regions of interest found by fluorescence with subsequent serial block face SEM analysis [7], while a combination of fluorescence backfilling with fluorescently labeled biocytin and metallographic contrasting using enzyme metallography [53, 107] has been used to correlate fluorescence with serial block face SEM. A fundamental challenge with these methods is the development of probes small enough to penetrate the thick specimens typically analyzed by such methods, yet producing sufficient EM signal for detection. The successful application of peroxidase-based staining (EnzMet) indicates that the potential exists to apply FNG, which is of comparable size.

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Conflict of interest JFH is the President of and RDP is employed by Nanoprobes, Inc.

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