

## CHAPTER 4

# GOLD CLUSTER LABELS AND RELATED TECHNOLOGIES IN MOLECULAR MORPHOLOGY

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## 1. INTRODUCTION: WHY CLUSTER LABELS?

Although intensely colored, even the largest colloidal gold particles are not, on their own, sufficiently colored for routine use as a light microscopy stain: only with very abundant antigens or with specialized illumination methods can bound gold be seen.<sup>1</sup> Colloidal gold probes were developed primarily as markers for electron microscopy, for which their very high electron density and selectivity for narrow size distributions when prepared in different ways rendered them highly suited.<sup>2</sup> The widespread use of gold labeling for light microscopy was made possible by the introduction of autometallographic enhancement methods. In these processes, the bound gold particles are exposed to a solution containing metal ions and a reducing agent; they catalyze the reduction of the ions, resulting in the deposition of additional metal selectively onto the particles. On the molecular level, the gold particles are enlarged up to 30 – 100 nm in diameter; on the macroscale level, this results in the formation of a dark stain in regions containing bound gold particles, greatly increasing visibility and contrast.<sup>3</sup>

The applications of colloidal gold have been described elsewhere;<sup>4,5</sup> in this chapter, we will focus on the use of covalently linked cluster complexes of gold and other metals. A gold cluster complex is a discrete molecular coordination compound comprising a central core, or ‘cluster’ of electron-dense metal atoms, ligated by a shell of small organic molecules (ligands), which are linked to the metal atoms on the surface of the core. This structure gives clusters several important advantages as labels. The capping of the metal surface by ligands prevents non-specific binding to cell and tissue components, which can occur with colloidal gold.<sup>6</sup> Cluster compounds are more stable and may be used under a wider range of conditions. Unlike colloidal gold, clusters do not require additional macromolecules such as bovine serum albumin or polyethylene glycol for stabilization, and the total size of the label is therefore significantly smaller. Since the clusters considered in this chapter are generally less than 3 nm in diameter, this allows the preparation of probes that are much smaller than conventional immunocolloids, and cluster labeling can take advantage of the higher resolution and penetration available with smaller conjugates. Most importantly, while colloidal gold is adsorbed to its conjugate probe, clusters are conjugated by chemically specific covalent cross-linking. Therefore, the range of possible conjugate targeting agents includes any probe containing an appropriate reactive group. Clusters conjugates have been prepared with a wide variety of molecules that do not form

colloidal gold conjugates, including lipids, oligonucleotides, peptides, and other small molecules.<sup>7</sup>

In addition to the development of gold cluster labeling technology, this chapter will also review new developments in the related metallographic, or metal deposition, methods. This includes gold enhancement, in which gold rather than silver is selectively deposited onto gold particles. We will also describe some results obtained using another novel metallographic procedure, enzyme metallography, in which metal is directly deposited from solution by an enzymatic reaction. Because the original, and most widespread, use of metal cluster labels is in electron microscopy, many of the light microscopy methods described were developed as extensions of, or complements to electron microscopy methods, and demonstrate their greatest advantages when used with electron microscopy; therefore reference will also be made to the electron microscope methods used in the same studies, and the unique information that may be obtained from the correlation of both methods.

## 2. CLUSTER LABELING METHODS, AND RELATED TECHNOLOGIES

### 2.1. Metal cluster labels

The prototype gold cluster label is the undecagold cluster, which contains a core of 11 gold atoms coordinated by seven tris (aryl) phosphine ligands and three halides or pseudohalides; this is then linked to biological macromolecules or other targeting agents by means of a single reactive substituent on one of the coordinated phosphine ligands.<sup>8</sup> However, undecagold is very small and difficult to visualize microscopically. Much better results have been obtained using the larger, 1.4 nm Nanogold label.<sup>7,9,10</sup> Although small and faintly colored in comparison with larger colloidal gold, when Nanogold is combined with autometallography, it is transformed into one of the most sensitive visualization and detection methods available. Nanogold combined with silver or gold enhancement has been used to visualize single copies of target genes in *in situ* hybridization experiments,<sup>11-13</sup> and can detect as little as 0.1 pg of a target IgG on immunodot blots.<sup>8</sup> While these sensitivities are now equal to or greater than those of colloidal gold, Nanogold conjugates also penetrate much more deeply into tissue sections and access hindered

antigens much more effectively. Labeling has been observed at depths of up to 40 microns in tissue sections.<sup>14,15</sup> This makes gold cluster labeling useful for several methods for which colloidal gold has previously produced poor results.

## 2.2. Autometallography: Silver Enhancement

“Autometallography” is also called “electroless deposition”, “silver (or gold) enhancement”, or “silver (or gold) development”. It refers to the use of metal nanoparticles as “seeds” or nucleation centers which, under the right conditions, cause metal ions in solution to be reduced to metal in the zero oxidation state and deposit in layers on the seed particle. This can be extremely useful, since the seed particle now becomes larger and more detectable, thus improving sensitivity. Applications include making small gold nanoparticle immunoprobes visible by EM<sup>16</sup> and LM,<sup>17</sup> detection in gene or other arrays,<sup>18</sup> detection in gels and blots,<sup>19</sup> *in situ* hybridization,<sup>11-13</sup> sensitive diagnostic tests,<sup>20,21</sup> and preparation of larger nanoparticles.<sup>22</sup> Methods of detection of metal particles are likewise expansive, including simple light absorption, electron and x-ray scattering, Raman spectral enhancement,<sup>23</sup> interaction with fluorophores (quenching<sup>24</sup> and enhancement<sup>25</sup>), and since the metal is conductive other properties may be utilized such as changes in conduction or capacitance between electrodes.<sup>26</sup> The metals deposited can also be varied, and although silver enhancement is commonly used, gold, copper, nickel, platinum, and many other metals may be used which may be advantageous for particular applications. Electroless deposition is used to coat plastics with metals, prepare computer hard disks, and plate metals, such as silverware.

How does it work? The process is somewhat related to photography, where a silver halide crystal defect caused by light becomes a nucleation site for silver reduction. In the case of a metal nanoparticle, the metal surface is the nucleation site. A developing solution is required, minimally consisting of the metal ions (e.g., Ag<sup>+</sup>) and a reducing agent (e.g., hydroquinone). The reactions are shown in Figure 1. The electromotive potentials for these reactions are listed on the right, and the positive sum (+0.101) indicates it is a spontaneous reaction. However, these potentials assume standard conditions of concentration, and the potentials vary with pH. At

lower pH the reaction is very slow. The metal particle surface is presumably catalytic by deforming the hydroquinone to reduce the activation energy and increase the rate of reaction. What size particle is necessary? It appears that 4 metal atoms is sufficient.<sup>27</sup> At the other end of the size range, what is the largest size? Of course the percent change in particle diameter with large particles is less for the same deposition rate, so upon enhancement, the small particles appear to “catch up” with the larger ones, leading to a more homogeneous size distribution, in some cases ~80nm.

What are some of the drawbacks of this system?

*Light sensitivity:* Some silver salts are light sensitive (e.g., silver lactate) and development should be in the dark, whereas silver acetate appears to be rather light insensitive.<sup>28</sup> Although some light sensitivity has been observed, useful reactions can be carried out in room lighting.

*Inhomogeneous particle sizes:* Gold nanoparticles usually have some organic coating, or are bound to biomolecules, and giving variable catalytic surfaces leading to different rates of deposition. Microscopically, some particles do not even develop at all. Additives to the developing mix, such as gum Arabic, and the speed of the development affect the final size distribution.

*Autonucleation:* the enhancement solution by itself will form nanoparticles that are then catalytic for further growth. This is due to the exothermic reaction conditions and nucleating impurities like small particles. This process can be delayed by various additives, or purification of reagents. It also creates a window for specific enhancement, since this non-specific metal deposition will ultimately ruin any specific reaction. One must be careful not to allow the enhancement to go too long, into this autonucleation zone. A useful method to obtain some additional sensitivity without autonucleation is to apply the developer for a period shorter than the autonucleation time, rinse it off, then apply freshly mixed developer, that starts a new autonucleation cycle. This can be repeated several times. Since developers will eventually autonucleate, they should be used immediately after mixing their components.

*Halides:* Silver ions precipitate with halides, so thorough washing with deionized water is required before enhancement.

*Temperature:* Higher temperatures usually lead to more rapid autonucleation times and too short a window for best use.

### 2.3. Gold enhancement

Although silver enhancement has traditionally been the most popular developer for gold particles, gold enhancement<sup>29,30</sup> has a number of advantages: (a) the pH is near 7, whereas many silver developers operate at ~pH 3.5; (b) size distribution of the enhanced particles is sometimes narrower than with silver; (c) the product is gold, which is much better for backscatter detection by SEM, or other applications;<sup>31</sup> (d) the gold product is chemically inert and not dissolved by oxidizing agents; for example, osmium tetroxide can dissolve silver but not gold;<sup>32</sup> (e) the reaction is more selective – for example, gold enhancement can be carried out in cells cultured on metal substrates;<sup>32</sup> and (f) the reaction may be used in the presence of chloride ions, useful for biology; silver ions form a precipitate with chloride but gold ions do not. Gold and silver enhancers are available from Nanoprobes, Inc. ([www.nanoprobes.com](http://www.nanoprobes.com)).

### 2.4. Combined fluorescent and gold probes

The control that covalent labeling provides over probe architecture and configuration has led to the development of another novel class of reagents, combined fluorescent and gold probes. Selective coupling of the gold cluster to a unique site in an antibody fragment, such as a hinge thiol, allows the attachment of a fluorescent label elsewhere on the antibody via a second cross-linking reaction, to yield a probe with both fluorescent and gold labels. Combined fluorescent and gold probes, available commercially as “FluoroNanogold” (Nanoprobes, Yaphank, NY, USA), may be used for correlative fluorescence and electron microscopy,<sup>33-37</sup> or for checking labeling by fluorescence microscopy before undertaking electron microscopy processing.

An important consideration when designing such probes is fluorescence resonance energy transfer. The overlap between the emission spectrum of the fluorescent label, and the absorption spectrum of the gold particle, allows for significant non-radiative energy transfer from the fluorescent label to the gold cluster, and reduces fluorescence intensities, as described by Förster.<sup>40</sup> Fortunately, this process is highly dependent upon the separation of the two labels:

provided they are positioned far enough apart, enough fluorescent emission remains for microscopic use. Calculations with the Nanogold cluster yield a Förster distance (the separation at which 50 % of the native fluorescence is retained) of approximately 6 nm, allowing usable fluorescence when both labels to be linked to a single antibody. However, extinction coefficients, and therefore overlap integrals and Förster distances, increase significantly for larger colloidal gold particles. Förster calculations indicate, for example, that for fluorescein and a 20 nm colloidal gold particle, the Förster distance is about 25 nm.<sup>41</sup> Although a few reports have described the preparation of combined fluorescent and colloidal gold probes, recent more rigorous studies using ultracentrifugation and pelleting to separate the gold conjugate from dissociated antibody confirmed that almost complete quenching of up to 99 % or more occurs in combined 6 and 18 nm colloidal gold and fluorescent conjugates: fluorescence was only obtained from the supernatant, confirming that the fluorescence signal actually arises from fluorescently labeled probes which have dissociated from the gold particles. Conjugation of the fluorescent label, in this case an Alexa Fluor 488, to a second antibody which binds to the gold-conjugated one, increases the gold-fluorophore separation to 10 – 20 nm; although quenching was still present, sufficient fluorescence was restored for observation by CCD camera, confirming the relationship between fluorescence quenching and separation.<sup>42</sup>

## **2.5. Nanogold-Autometallography**

Aside from the general procedures and properties of silver and gold enhancement of metal particles, there are more specific considerations and advantages of using small Nanogold particles as nucleating centers for the deposition reactions. The 1.4 nm diameter Nanogold (diameter of solid gold core; 2.7 nm total diameter with phosphine shell), is considerably smaller than the commonly used 10-40 nm gold. The advantages of using a smaller nanoparticle include: 1) better penetration into tissue, 2) less steric hindrance, so that more epitopes are labeled, 3) the linkage of Nanogold to antibody is covalent and more stable than antibodies just adsorbed to 10 nm gold, 4) better washing of unbound gold, 5) there may be less background binding, and 6) small ligands, peptides, or compounds may be stably attached to the gold,<sup>9,10</sup> whereas small

molecules usually do not adsorb stably to colloidal gold. The smaller gold particles are not as visible initially, but after enhancement they produce a clear signal, either at the EM, LM, or unaided eye level. So, after enhancement of a 10 nm colloidal gold immunoprobe vs. a Nanogold immunoprobe, which gives the strongest signal? It is our experience, using dot blots as a test, that the Nanogold conjugate, although weaker without enhancement, is more sensitive by an order of magnitude or more after they are both enhanced with silver or gold. Many studies have now been published using Nanogold for immunolocalization. Gold is the preferred marker for electron microscopy (EM), so Nanogold is ideal for that field. However, with further metal enhancement, the advantages of using small covalent gold can be appreciated in tissue applications at the LM level. Takizawa and Robinson showed that Nanogold-Fab' probes penetrated fully into 2  $\mu\text{m}$  cryosections, whereas 5 nm colloidal golds hardly penetrated, and 10 nm colloidal gold-IgG probes did not enter at all.<sup>14</sup> 2  $\mu\text{m}$  cryosections were re-cut perpendicularly so that they could be viewed from the side to see the extent of penetration. Due to the far denser labeling, intense staining was observed with Nanogold. They noted that DAF, a relatively low-abundance protein in neutrophils, was difficult to detect with 10 nm gold, but readily visualized with Nanogold-Fab'.

Since the Nanogold particle is so small (1.4 nm), for most applications (except high deposition or high resolution EM), it needs to be silver or gold enhanced for best visibility.

Detection of cellular components can be done using a directing moiety that binds to the desired target. The targeting moiety can be an antibody, antibody fragment, peptide, protein, nucleic acid, drug, carbohydrate, lipid, charged/hydrophobic/hydrophilic group, or other ligand. Nanogold has a monofunctional linking arm that provides standard covalent crosslinking chemistry to attach to amines or thiols, or other groups if required. The smallest antibody probe, giving excellent penetration, was formed by linking Nanogold to recombinant Fv antibody fragments.<sup>43</sup>

Several examples of enhanced gold in studies of human tissues are provided in the review by Lackie.<sup>44</sup>

## 2.6. Double labeling

Nanogold may be used in double labeling experiments, so that two labels can be visualized together. For electron microscopy, two strategies are as follows. One is to silver enhance Nanogold, but make it distinguishable from some standard colloidal gold sizes. This was done by Takizawa and Robinson<sup>14</sup> where Nanogold, because of its better sensitivity over colloidal gold, was used to detect a low abundance epitope. After silver enhancement, a 10 nm colloidal gold immunoconjugate was used to label the second target. Two other examples used Nanogold in double labeling with 30 and 40 nm colloidal gold in neurological tissue to distinguish receptors.<sup>45,46</sup> A second strategy is to silver develop the first target gold particle to larger size, then label the second target with gold and apply a second silver enhancement.<sup>47</sup> Since the first gold particles will then have been developed twice, they become distinguishably larger than the second label.

## 2.7. Enzyme metallography

Oxidoreductases such as horseradish peroxidase are commonly used to generate colors for detection, e.g., soluble NBT/BCIP, or insoluble DAB. Tissue immunohistochemistry frequently relies on the DAB reporter. A more recent alternative developed by Nanoprobes is the enzymatic deposition of gold nanoparticles that serve as substrates.<sup>48</sup> These produce intense metal deposits that enhance viewing and also extend the utility to EM, where the metals are clearly visible, whereas organic deposits, such as DAB are not.

More recently, it has been found that redox enzymes, in particular horseradish peroxidase (HRP) can also catalyze the selective deposition of metal from solution. Further investigation revealed that this reaction could be used for highly specific and sensitive staining both for immunohistochemistry and *in situ* hybridization,<sup>49</sup> and in some cases produced cleaner, darker staining than that using gold cluster labeling with autometallography.<sup>50</sup> Preliminary results indicate that this method, like cluster labeling, also produces specific staining in the electron microscope, and thus may be applicable as a correlative light and electron microscopic stain.<sup>50</sup>

An interesting observation about this technology is that the deposits are very well localized, whereas DAB is known to spread away from the site of origination before becoming fixed, leading to a lower resolution signal. Immunohistochemical and in situ hybridization detection using enzyme metallography are shown in Figure 2.

### 3. SPECIFIC GOLD CLUSTER PROBES AND METHODS

#### 3.1. LI Silver Enhancement of Nanogold Conjugates in Light Microscopy

If aldehyde-containing reagents have been used for fixation, these must be quenched before labeling. This may be achieved by incubating the specimens for 5 minutes in 50 mM glycine solution in PBS (pH 7.4); ammonium chloride (50 mM) or sodium borohydride (0.5 - 1 mg/ml) in PBS may be used instead of glycine. To prepare the developer, mix equal amounts of the enhancer and initiator immediately before use. Nanogold will nucleate silver deposition resulting in a dark staining depending on development time. Additional steps, such as postfixing, may be used as required. Optimum results should be obtained using the buffers and washes specified in the instructions for the Nanogold reagents.

1. Spin cells onto slides using Cytospin, or use paraffin section.
2. Incubate with 1 % solution of bovine serum albumin in PBS (PBS-BSA) for 10 minutes to block non-specific protein binding sites.
3. Incubate with primary antibody, diluted at usual working concentration in PBS-BSA (1 hour or usual time)
4. Rinse with PBS-BSA (3 X 2 min).
5. Incubate with Nanogold reagent diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum from the same species as the Nanogold reagent, for 1 hour at room temperature.
6. Rinse with PBS (3 X 5 min).
7. Postfix with 1 % glutaraldehyde in PBS at room temperature (3 mins).

8. Rinse with deionized water (3 X 1 min).
9. Develop specimen with freshly mixed developer for 5-20 minutes. More or less time can be used to control intensity of signal. A series of different development times may be used, to find the optimum enhancement for your experiment; generally a shorter antibody incubation time will require a longer silver development time.
10. Rinse with deionized water (2 X 5 mins).
11. The specimen may now be stained if desired before examination, with usual reagents.

**PBS-BSA Buffer:**

20 mM phosphate  
150 mM NaCl  
pH 7.40  
0.5% BSA  
0.1% gelatin (high purity)

**PBS Buffer:**

20 mM phosphate  
150 mM NaCl  
pH 7.40

*Optional, may reduce background:*

0.5 M NaCl  
0.05% Tween 20

### **3.2. Silver Acetate Autometallography of Nanogold Conjugates for Light Microscopy**

Silver acetate autometallography has been found to be highly sensitive and specific for in situ hybridization with Nanogold. Detailed protocols for specific procedures using this method are available online from the Research Institute for Frontier Questions of Medicine and Biotechnology (<http://www.frontierquestions.com/labprotocols.htm>). Solutions A and B should be freshly prepared for every run. To prepare the enhancement solution: Just before use, mix solution A with solution B.

1. Deparaffinize sections and bring to water through graded alcohols.

2. Distilled water (> 3 min).
3. Antigen retrieval as appropriate.
4. Oxidize in Lugol's iodine solution (Merck) (5 min).
5. Rinse in tap water (> 10 sec), then distilled water (> 10 sec).
6. Reduce in 2.5% sodium thiosulfate solution till colorless (a few sec).
7. Wash thoroughly in tap water (> 10 sec), then in distilled water (2 min).
8. Drain off, wipe-dry area around section and apply DAKO-pen.
9. Immerse in PBS containing detergent (> 5 min).
10. Apply 1:20 normal serum of the species providing the secondary antibody (5 min).
11. Drain off and incubate with appropriately diluted primary antibody (overnight at 4°C).
12. Wash in PBS-gelatin, also containing detergent (3 x 5 min).
13. Incubate with biotinylated anti-rabbit or anti-mouse immunoglobulins (DAKO, 1:200 in PBS) (30 min at RT).
14. Wash in PBS-gelatin, also containing detergent (3 x 5 min).
15. Incubate with streptavidin-Nanogold diluted 1:750 in PBS-BSA (60 min at RT).
16. Wash in PBS-gelatin, also containing detergent (3 x 5 min).  
Avoid the use of metal forceps from this step on!
17. Wash repeatedly for at least 15 min in Ultrapure water and then apply silver enhancement (silver acetate autometallography). Place the slides vertically in a glass container (preferably with about 80 mL volume and up to 19 slides; Schiefferdecker-type) and cover them with the mixture of solutions A and B. Staining intensity can be checked in the light microscope during the amplification process, which usually takes about 5-20 min, depending on primary antibody or nucleic acid probe concentration, incubation conditions, and the amount of accessible antigen or nucleic acid sequence in question.
18. Rinse carefully.
19. Counterstain with H&E or Nuclear Fast Red, dehydrate and mount in DPX or Permount. Avoid Eukitt.

**Solution A:**

Dissolve 80 mg silver acetate (code 85140; Fluka, Buchs, Switzerland) in 40 mL of glass double-distilled water.

(Silver acetate crystals can be dissolved by continuous stirring within about 15 min.)

**Citrate buffer:**

Dissolve 23.5 g of trisodium citrate dihydrate and 25.5 g citric acid monohydrate in 850 mL of deionized or distilled water. This buffer can be kept at 4°C for at least 2-3 weeks.

Before use, adjust to pH 3.8 with citric acid solution.

**PBS:**

Phosphate-buffered saline (PBS) pH 7.6 containing 0.1 % Tween 20 or Triton X-100.

**PBS-gelatin:**

BS containing 0.1% fish gelatin (Sigma).

**Solution B:**

Dissolve 200 mg hydroquinone in 40 mL citrate buffer (below).

**PBS-BSA:**

PBS pH 7.6 containing 1% bovine serum albumin.

### 3.3. Gold Enhancement for Light Microscopy

GoldEnhance is prepared immediately before use by mixing equal amounts of Solution A (enhancer) and Solution B (activator), followed by the Solution C (initiator), and Solution D (buffer). For optimum results, we recommend waiting 5-10 minutes after mixing A and B before adding C and D, although the reagent will produce successful enhancement if C and D are added

immediately to up to two hours later. The reagents are supplied in dropping bottles for easier dispensing of small amounts.

If aldehyde-containing reagents have been used for fixation, it is recommended that these be quenched before labeling. This may be achieved by incubating the specimens for 5 minutes in 50 mM glycine solution in PBS (pH 7.4); ammonium chloride (50 mM) or sodium borohydride (0.5 - 1 mg/ml) in PBS may be used instead of glycine.

The following procedure was developed for gold enhancement of In Situ hybridization specimens by Hacker et al. as a modification of the Nanogold-Silver Staining procedure. (Hacker et al., 1997). It has been found to be effective for enhancement of tissue sections for light microscope observation. We have found times of 10-20 minutes give optimal results; however, this reagent is intended to function in a wide range of conditions, and different washes and development times may give better results in your application. You should follow your normal procedure up to the application of the gold conjugate; the protocol below describes the steps after this:

1. Incubate the sections with Nanogold or colloidal gold conjugate according to current protocols or using the buffers, concentrations and protocols recommended for the conjugate.
2. Wash in PBS pH 7.6, 2 times 5 min each.
3. Wash in PBS-gelatin pH 7.6 for 5 min.
4. Repeatedly wash in distilled water for at least 10 min altogether, the last 2 rinses in ultrapure water (EM-grade).
5. Prepare GoldEnhance using equal amounts of the four components (Solutions A, B, C, and D); prepare about 80  $\mu$ L per slide.
6. Dispense Solution A (enhancer: green cap) into a clean tube or dish, add Solution B (activator: yellow cap), and mix thoroughly.
7. Wait 5 minutes.
8. Add Solution C (initiator: purple cap) and Solution D (buffer) and mix thoroughly.
9. Apply 1-2 drops (about 80 microliters, sufficient to cover the specimen) to the slide.
10. Develop specimen for 10 - 20 minutes. More or less time can be used to control particle size and intensity of signal.

11. When optimum staining is reached, immediately stop by rinsing carefully with deionized water.

**PBS-Gelatin Buffer:**

20 mM phosphate

150 mM NaCl

pH 7.6

**PBS Buffer:**

20 mM phosphate

150 mM NaCl

pH 7.6

*optional, may reduce background:*

0.1% gelatin (high purity)

0.5 M NaCl

0.05% Tween 20

To obtain an especially dark signal, gold enhancement may be repeated with a freshly mixed portion of GoldEnhance.

**Notes:**

- Development starts with addition of Solution C (initiator), so apply to sample as soon as possible after adding C and D to minimize autonucleation background.
- To obtain an especially dark signal, or for further development, develop longer or gold enhancement may be revitalized with a freshly mixed portion of GoldEnhance (rinse with distilled after between applications of GoldEnhance).
- The development is not highly light sensitive, so may be conducted under normal room lighting, or viewing by light microscopy.
- Some users reported good development omitting the use of Solution D, but deposition times are then slower.

### **3.4. Correlative Light and Electron Microscopy**

Nanogold and related smaller gold labels are the preferred labels for EM due to their small size, high electron scattering, and high resolution with which they bind targets. However, because Nanogold may be silver or gold developed to produce large particles or coalesced aggregates that are visible by LM, it may be used for correlative microscopy: it is possible to carry out a single labeling experiment to label both at the EM and LM level and thus to provide direct correlation between the two. This is extremely useful, since if a light microscopic experiment is done with one probe, such as a fluorescent antibody probe, and the other with a colloidal gold conjugate, the results often have been found to be inconsistent. Discord can arise from differences in penetration, binding constants, probe purity (e.g., free antibody will alter results), efficiency of binding to antigen, washout rates, antibody differences, and different background binding characteristics. A more direct and consistent approach is to use one optimal probe, and examine its distribution both by EM and LM, even in the same cell. This is possible using Nanogold and altering the time of enhancement. An example of correlative LM/EM with silver enhanced Nanogold is the work by Sun et al.<sup>15</sup> where labeling in thick sections examined by laser scanning confocal microscopy (LSCM) was matched with EM sections. Neurons were labeled with neurobiotin or biocytin, Vibratome sectioned, stained with Nanogold-streptavidin (for EM), and Cy3-streptavidin (for LM) and embedded in epon/araldite. LSCM optical sectioning was used to find interesting areas, then these were directly thin sectioned for EM. They found the Nanogold-streptavidin reliably labeled up to 40  $\mu\text{m}$  into the tissue. Takizawa and coworkers also immunolocalized epitopes and found them in the same cell both by light and electron microscopy.<sup>36</sup>

### **3.5. Gold Cluster Labels for Chromogenic Microarray and Biochip Detection**

Gene arrays and chips are becoming more popular for screening tissues and cells to discover genetic differences by comparing extracted DNA, and to find up and down regulation by examining mRNA. Target sequences are initially placed in spots or micro squares, the sample is

applied, and hybridization detected by a reporter. Although fluorophores are commonly used, metal nanoparticles have many advantages. The signal does not fade or change over time or with observation as fluorescence does, and detection is simpler, not requiring fluorescent optics. Alexandre and co-workers have reported that Nanogold and silver enhancement functions as a simple, low-cost colorimetric detection method for DNA microarrays: a biotinylated target, human cytomegalovirus DNA, was detected after capture using Nanogold-streptavidin followed by silver enhancement by sequential application of silver nitrate followed by hydroquinone. Signals were read using an array colorimetric workstation comprising a computer tower equipped with a CCD camera, illumination and image analysis software. The sensitivity of this method was found to be equal to that found using Cy3 fluorescence detection, corresponding to 1 amol of biotinylated DNA attached on an array.<sup>18</sup> The high sensitivity of visualization and optical detection enabled by autometallography also makes this method suitable for use in other optical detection systems, such as biochips.

### **3.6. Gold Cluster-Labeled Lipids**

Since gold clusters or nanoparticles may be attached to almost anything (DNA, proteins, drugs, carbohydrates), why not lipids? Nanogold is a good candidate for covalently linking to lipids, since it is monofunctional and provides standard chemistry for making a stable covalent conjugate. The gold particle is attached to a suitable reactive functionality positioned in the hydrophilic head-group of the lipid; the dangling, hydrophobic tail is then free to insert into liposomes or other lipid-based structures. Nanogold conjugates are available with phospholipids or fatty acids, and the structures are shown in Figure 3.

These molecules have been used to form monolayers on air-water interfaces, creating arrays of gold particles. Gold-decorated liposomes, or “metalloosomes” may be prepared by sonication of the gold-labeled lipids, either alone or mixed with unlabeled lipids, in water, and a number of different morphologies have been found for the resulting vesicles.<sup>52</sup>

These probes may also be used to track liposomal species in the body. Adler-Moore used this to monitor delivery and action of the anti-fungal channel-forming drug, amphotericin: this is

most effective in a liposomal formulation where it is incorporated into 45 – 80 nm liposomes composed of hydrogenated soy phosphatidylcholine, distearoyl Phosphatidylglycerol and cholesterol, which reduced preclinical toxicity 30-fold in rodents. Fluorescently labeled liposomes both with and without the drug were localized by fluorescence microscopy *in vitro*, and in sections of kidney taken from mice challenged with *Candida albicans* and subsequently injected with liposomes. Fluorescence studies showed localization of the drug-containing liposomes to the fungal cells in the mouse kidney and penetration of the fluorescent signal into the interior of fungal cells *in vitro*; resonance energy transfer (RET) studies showed that the constituents of drug-bearing liposomes were dispersed upon fungal penetration, suggesting that the fungal cell wall was disrupted by the drug. This was confirmed by electron microscopy studies using liposomes spiked with a small amount of dipalmitoyl phosphatidyl ethanolamine (DPPE)-Nanogold: after a 14 hour incubation with *Aspergillus fumigatus* (one of the drug targets), Nanogold delivered in non-drug-bearing liposomes was almost entirely incorporated into the fungal cell wall, while the Nanogold delivered in amphotericin-loaded liposomes had entered the fungal cells and was distributed throughout the cytoplasm.<sup>53</sup> DPPE-Nanogold with silver enhancement was also used to label cationic liposomes and elucidate their targeting to endothelial cells in tumors and areas of chronic inflammation in mice.<sup>54</sup>

### **3.7. Localization and Detection of DNA: *In Situ* Hybridization and *In Situ* Pcr**

The staining produced by gold cluster labeling has several characteristics that make it ideal for *in situ* hybridization. The small size of gold clusters, particularly since they do not require additional macromolecules for stabilization and do not aggregate to form larger oligomers, means that they penetrate readily into cells and tissue sections to access nuclear targets. The staining produced by autometallographically enhanced gold is black, highly opaque, and punctate, and is therefore visually distinct from commonly used counterstains. The punctate nature of staining means that the signals are also highly localized; while this is not significant for some light microscopy applications, it means that should the need arise for subsequent electron microscopy, the same staining may be visualized at the higher resolution obtainable with EM.

Most importantly, the sensitivity of this method and modifications towards low copy number targets means that it may reliably be used to detect even genes present in only one or two copies.

Although well-defined staining could be achieved for abundant targets, such as HPV16 in CaSki cells, which contain several hundred copies, by direct *in situ* hybridization with a biotinylated cDNA probe followed by detection with Nanogold-streptavidin and enhancement using a silver acetate developer, for the detection of very low copy number targets, a target or signal amplification step is required. While both approaches have been used, in the systems studied most widely, target amplification, which is achieved using *in situ* PCR,<sup>55</sup> was found to be less specific than signal amplification due possibly to diffusion of the amplified targets. Therefore most of the subsequent work has utilized signal amplification, using tyramides or similar methods.

A variety of different protocols have been investigated using different haptens, signal amplification schemes, and autometallography methods to optimize and simplify this method. In the original work, Hacker demonstrated consistent detection of HPV16/18 in SiHa cells, known to contain only one to a few copies of the target gene, using a fluorescein-conjugated DNA probe or riboprobe, which was detected using a biotinylated secondary antibody which was in turn detected with avidin-biotin-complex and biotin-HRP followed by treatment with biotin-tyramide and ultimately detection using Nanogold-streptavidin followed by enhancement with silver acetate developer.<sup>56</sup> Later studies were simplified by the use of a biotinylated hybridization probe and HRP-streptavidin to deposit biotinylated tyramide.<sup>57</sup>

Application of the gold enhancement process to *in situ* hybridization has resulted in the development of protocols that are sufficiently sensitive and reproducible to find applications in diagnostic pathology. Chromogenic-*in situ* hybridization methods have attracted a great deal of attention recently, and a consensus has developed that they offer important advantages for the practicing pathologist. Unlike fluorescent methods, they do not require expensive fluorescence optics, or require dark adaptation on the part of the user; instead, staining may be interpreted using a standard brightfield light microscope. The underlying cell and tissue morphology is visualized simultaneously with the target, yielding additional information that may be helpful for diagnosis; and the autometallographically enhanced staining is permanent. Tubbs describes the development of a gold-facilitated *in situ* hybridization (GOLDFISH) assay for the detection of HER2 gene amplification in breast carcinoma;<sup>58</sup> this method correlated very well with

fluorescent in situ hybridization (FISH) in a series of 104 clinical cases,<sup>59</sup> and a simplified interpretation procedure was developed in which positive and negative results were differentiated by the degree of obscuration of the nucleus rather than the number of spots. A number of enzyme chromogenic *in situ* hybridization studies of the same system have been described, and both methods are reported to yield a similar degree of accuracy.<sup>60-62</sup>

Although GOLDFISH produces excellent correlation with FISH and the simplified interpretation speeds up diagnosis, the interpretation does not fully resolve the important issue of interpreting whether 'low-level' amplification is clinically significant. In order to do so, a cut-off value must be established for the number of gene copies that constitute genuine low-level amplification; 5 and 6 have been proposed. Identifying such cases unambiguously requires a return to interpretation by spot counting; fortunately, image analysis methods are now available for the automation of this process. Enzyme metallography was found to be highly effective for such studies, and also represents a further simplification of the procedure: a biotinylated probe is detected using HRP-streptavidin, then treated with the metallographic substrate. Individual gene copies were sharply distinguished, supporting spot counting both manually and potentially by automated image analysis and clearly visible against counterstains.<sup>50</sup> In addition, staining was found to be even cleaner and backgrounds even lower using the new method. The most recent modification of this method, SILVERFISH, includes the simultaneous assessment of the concomitant HER2 protein CB11 antibody and Fast Red K stain, thus providing an internal confirmation of protein overexpression.<sup>63</sup>

### **3.8. Combined Fluorescent and Gold Labeling Applications**

Combined fluorescent and gold probes bring the light microscopy and electron microscopy together even more forcibly than cluster labels alone, since the principal reason for having both labels are to obtain information from both using a single probe. Early studies showed that cellular penetration was not reduced by the attached fluorescent label, illustrated by labeling of the SC35 pre-mRNA splicing site in HeLa cells.<sup>34</sup> These new probes could be imaged using many microscope modalities, allowing many combinations of data. Robinson, Takizawa and co-

workers used them to label and image microtubules in human leukocytes, a system that yielded very poor and erratic labeling with colloidal gold: the microtubules could be visualized, after a brief 1-2 minute silver enhancement, by fluorescence, standard brightfield, phase contrast, differential interference and epifluorescence microscopy.<sup>35</sup> Later studies using locator grids yielded images of the same sites by fluorescence and transmission electron microscopy, demonstrating proof of principle for correlative labeling.<sup>36</sup>

Combined Nanogold and fluorescent-labeled Fab' antibody probes were prepared by the initial reaction of a hinge thiol group, obtained by selective reduction of the hinge disulfide in F(ab')<sub>2</sub> fragments, followed by reaction with maleimido-Nanogold, followed by reaction of the gold-labeled conjugate with amine-reactive fluorophores. This method was later applied to the preparation of combined Cy3 and Nanogold-Fab' probes, which were used to image the polar tubes in microsporidia by fluorescence and transmission electron microscopy,<sup>37</sup> and recently to combined Nanogold and Alexa Fluor 488<sup>64</sup> or 594 conjugates.

Reconciling desired sensitivity and labeling density with control over background binding initially represented a significant challenge in the use of combined fluorescent and gold probes. Because fluorescent probes often require higher concentrations to achieve acceptable labeling than immunogold probes, finding the optimum concentration for combined fluorescent and gold labeling may require a compromise. In addition, inclusion of the fluorescent label adds can add a new hydrophobic or ionic character to the probe, which may be a mechanism for background binding. However, a number of methods have been developed to reduce this. Spector and co-workers found, after a comparison of commonly used buffers, that washing with sodium citrate immediately before silver enhancement reduced background signal effectively in HeLa cells: 0.02 M sodium citrate at pH 7.0 was most effective for silver enhancement using the Danscher formulation, while 0.02 M sodium citrate at pH 3.5 was most effective before application of HQ Silver (Nanoprobes).<sup>34</sup> More generally, the addition of 5 % nonfat dried milk, either as a blocking step before addition of the combined fluorescent and gold probe, or included in the incubation buffer in which this probe is applied, has been found to greatly reduce non-specific fluorescence signal.

Combined Nanogold and fluorescently labeled streptavidin was prepared by reaction with Mono-*Sulfo*-NHS-Nanogold; after isolation of the gold conjugate, a second conjugation was undertaken with an amine-reactive derivative of the fluorophore. Combined Nanogold and

fluorescein, Cy3<sup>37</sup> and Alexa Fluor 488 and 594 streptavidin<sup>38,39</sup> have been prepared in this manner. The combined Cy3 and Nanogold labeled streptavidin was used for the The combined Cy3 and Nanogold-labeled streptavidin was used for the in situ detection of HPV-16 viral DNA in CaSki and SiHa cells, using the same methods described previously for Nanogold-silver staining. Using a DNA probe with tyramide signal amplification, bright fluorescent signals were obtained in CaSki cells, which contain several hundred copies of the genetic target; however, clear signals were obtained even in SiHa cells, which contain only one to a few copies of the viral DNA, produced clear fluorescence signals. Detection was confirmed by gold enhancement followed by brightfield light microscope observation, which duplicated the signal localization; as a control, when the biotinylated tyramide reagent was omitted, neither fluorescent nor gold-gold brightfield signals was observed. Signal localization in CaSki cells was confirmed by electron microscopy.<sup>37</sup>

Recently, Robinson and Takizawa have described the use of combined Alexa Fluor 594 and Nanogold-labeled streptavidin for correlative localization of caveolin-1 in ultrathin cryosections of terminal villi of the human term placenta by fluorescence and immunoelectron microscopy. The use of ultrathin cryosections enabled high spatial resolution by fluorescence microscopy because there is essentially no out-of-focus fluorescence. Electron microscopy immunolabeling obtained with colloidal gold and the combined Alexa Fluor and Nanogold probe were compared using a particle counting procedure: a higher number of particles was found with silver-enhanced Alexa Fluor and Nanogold than with colloidal gold.<sup>38,39</sup>

Tissue was cut into small pieces, fixed in freshly prepared 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 5% sucrose for 2 hr at room temperature, then washed and embedded in 10% gelatin in the same buffer. The solidified gelatin was cut into smaller pieces and then cryoprotected by infiltration with 2.3 M sucrose in 0.1 M sodium cacodylate (pH 7.4) overnight at 4°C. Ultrathin cryosections (100-nm thickness or less) were collected on droplets of 0.75% gelatin-2.0 M sucrose or 1 % methylcellulose-1.15 M sucrose, and transferred to nickel Maxtaform “finder” grids (Graticules; Tonbridge, Kent, UK) to facilitate re-location of specific fluorescently labeled features for EM processing. Sections were immersed in a solution containing 1% non-fat dry milk and 5% fetal bovine serum in PBS (MFBS-PBS) for 15 min at 37°C to remove the sucrose and gelatin, then washed three times in PBS and incubated in MFBS-PBS with 0.05 % sodium azide to block nonspecific protein

binding. The grids were incubated with biotin-labeled goat anti-chicken (13 g/ml in MFBS-PBS) for 30 min at 37°C, washed in PBS for 12 min with four changes, immersed in MFBS-PBS, then incubated with Alexa Fluor 594 FluoroNanogold-streptavidin (diluted 1:50 in MFBS-PBS) for 30 min at room temperature. The grids were then washed in PBS for 15 minutes with five changes and mounted on a glass microscope slide in PBS containing 1% N-propyl gallate and 50% glycerol, pH 8.0, to retard photobleaching, and coverslipped. After optical microscopy and noting regions of interest on the “finder” grid for relocation in the electron microscope, the temporary slide preparations were then disassembled and the grids washed in PBS with five changes over 15 min. The ultrathin cryosections were then fixed in 2% glutaraldehyde in PBS for 30 min, washed in distilled water for 6 min with four changes, and dried with filter paper. The grids were then floated on drops of distilled water and subsequently on drops of 50 mM 2-[N-morpholino]ethanesulfonic acid buffer, pH 6.15, for 4 min with two changes, followed by silver enhanced using a freshly prepared N-propylgallate-based silver enhancement formulation for 3 minutes. The same regions examined by fluorescence microscopy were relocated and electron micrographs collected.

Elizabeth Schroeder-Reiter and colleagues have reported high-resolution detection and localization of nuclear features by correlative fluorescence and scanning electron microscopy. Phosphorylated histone H3 at serine 10 in mitotic barley chromosomes isolated and mounted either on laser-marked glass slides or on standard glass slides. Slides were incubated in PBS, blocked with 1% bovine serum albumin in PBS for 30 min, then incubated with primary antibody, (polyclonal rabbit antibody against histone H3 phosphorylated at serine position 10) diluted 2 : 500 in the blocking solution for one hour; after washing in PBS, the labeled secondary antibody was applied for one hour. The slides were subsequently washed in PBS, and specimens postfixed with 2% glutaraldehyde in PBS. Immunogold-labeled specimens were washed with distilled water, enhanced either with GoldEnhance or HQ Silver, then washed in 100% acetone and critical point dried. Slides were first controlled with LM in phase contrast mode, then carbon-coated by evaporation to a layer of 3 - 5 nm and examined at an accelerating voltage of 12 -15 kV. Back-scattered electrons (BSE) were detected with a YAG-type detector (Aurata); secondary electron (SE) and BSE images were recorded simultaneously. Nanogold, FluoroNanogold, and 10 nm colloidal gold secondary immunoprobables were compared: while a 10 nm colloidal gold conjugate gave poor labeling and lack of correlation with fluorescent signals,

both Nanogold and FluoroNanogold produced dense labeling which correlated well with both fluorescence labeling and known target distribution.<sup>65</sup>

In addition to the combined Nanogold and fluorescently labeled immunoprobes and streptavidin, a number of other combined fluorescent and gold labeled probes have been reported. Antibody Fab' fragments labeled with 1.8 nm platinum clusters and either fluorescein or Texas Red, were found to give detectable labeling of red blood cells;<sup>66</sup> Robinson's' finding that fluorescence is still visible even after brief silver enhancement of the combined fluorescein and Nanogold probes confirms that larger cluster labels may feasibly be used to prepare combined fluorescent and gold probes.<sup>35</sup> More recently, Texas Red and Mono-Sulfo-NHS-Nanogold were conjugated to a 10,000 MW amino-functionalized dextran; the resulting combined fluorescent and gold-tagged entity was used as a neuronal trace were used to retrogradely label spinal motor neurons innervating a median unpaired fin, the sexually dimorphic anal fin musculature in female and male Western Mosquitofish, *Gambusia affinis affinis*, in order to asses sex differences in spinal motor nuclei organization. The sexually dimorphic anterior transposition of the median unpaired fin, specifically the anal fin, provides a versatile experimental model for studying intercellular mechanisms, processes, and interactions during post-embryonic development, especially changes of the nervous system as the animal changes from a non-internal fertilizing to an internal fertilizing species. Retrograde tract tracing using this and other probes revealed a unique spinal cord region associated with the 12th through 14th vertebrae, a portion of the unique ano-urogenital region, containing a population of secondary motor neurons with extensive dendritic arborization; the female *G. a. affinis* was shown to have fewer and smaller secondary motor neurons than did males, and the neurons branching and dendritic arborization were more reduced than those in males.<sup>67</sup>

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#### 4. REFERENCES

1. Ackerley, C. A., unpublished results, 2000.
2. Handley, D. A., The development and application of colloidal gold as a microscopic probe, in *Colloidal Gold: Principles Methods and Applications*, Hayat, M. A., Ed., Academic Press, San Diego, 1989, vol. 1, chap. 1, pp. 1-12.

3. Danscher, G., Autometallography: A new technique for light and electron microscopic visualization of metals in biological tissues (gold, silver, metal sulphides and metal selenides), *Histochemistry*, 81, 331, 1984.
4. De Mey, J., Colloidal gold probes in immunocytochemistry, in *Immunocytochemistry - Practical Applications in Pathology and Biology*, Polak, J. M. and Van Norden S., Eds., Wright, Bristol, 82, 1983.
5. Bendayan, M., Worth its weight in gold, *Science*, 291, 1363, 2001.
6. Behnke, O., et al., Non-specific binding of protein-stabilized gold sols as a source of error in immunocytochemistry *Eur J Cell Biol*, 41, 326, 1986.
7. Hainfeld, J. F., Powell, R. D. and Hacker, G. W., Nanoparticle molecular labels, in *Nanobiotechnology*, Mirkin C. A. and Niemeyer, C. M., Wiley-VCH, Weinheim, Germany, 2003, chap. 23, pp. 353-386.
8. Hainfeld, J. F., Undecagold-antibody method, in *Colloidal Gold: Principles Methods and Applications*, Hayat, M. A., Ed., Academic Press, San Diego, 1989, vol. 2, chap. 21, pp. 413-429.
9. Hainfeld, J. F. and Furuya, F. R., A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling, *J Histochem Cytochem*, 40, 177, 1992.
10. Hainfeld, J. F. and Powell, R. D., New frontiers in gold labeling, *J. Histochem Cytochem.*, 48, 471, 2000.
11. Tubbs, R. R., et al., Supersensitive in situ hybridization by tyramide signal amplification and Nanogold silver staining: The contribution of autometallography and catalyzed reporter deposition to the rejuvenation of in situ hybridization, in *Gold and Silver Staining: Techniques in Molecular Morphology*, Hacker G.W. and Gu, J., Eds., CRC Press, Boca Raton, London, New York & Washington, D.C., 2002, chap. 9, pp. 127-144.
12. Tubbs, R., et al., Gold-Facilitated in Situ Hybridization: A Bright-Field Autometallographic Alternative to Fluorescence in Situ Hybridization for Detection of HER-2/neu Gene Amplification, *Am J Pathol*, 160, 1589, 2002.

13. Hacker, G.W., et al., In situ localization of DNA and RNA sequences: super-sensitive in situ hybridization using streptavidin-Nanogold-silver staining: Minireview, protocols and possible applications, *Cell Vision*, 4, 54, 1997.
14. Takizawa, T. and Robinson, J. M., Use of 1.4-nm immunogold particles for immunocytochemistry on ultra-thin cryosections, *J Histochem Cytochem*, 43, 1615, 1994.
15. Sun, X. J. Tolbert, L. P. and Hildebrand, J. G., Using laser scanning confocal microscopy as a guide for electron microscopic study: a simple method for correlation of light and electron microscopy, *J Histochem Cytochem*, 43, 329, 1995.
16. Scopsi, L., Silver-enhanced colloidal gold method, in *Colloidal Gold: Principles Methods and Applications*, Hayat, M. A., Ed., vol. 1, chap. 9, pp. 252-297.
17. Hacker G.W., et al., Silver staining techniques, with special references to the use of different silver salts in light- and electron microscopical immunogold-silver staining, in, in *Immunogold-Silver Staining: Principles, Methods and Applications*, M. A. Hayat, Ed., CRC Press, Boca Raton, FL, 1995, chap. 2, pp. 20-45.
18. Alexandre, I., et al., Colorimetric silver detection of DNA microarrays, *Anal Biochem*, 295, 1, 2001.
19. Hainfeld, J. F. and Furuya, F. R., Silver enhancement of Nanogold and undecagold, in *Immunogold-Silver Staining: Principles, Methods and Applications*, M. A. Hayat, Ed., CRC Press, Boca Raton, FL, 1995, chap. 5, pp. 71-95.
20. Chandler, J., Gurmin, T. and Robinson, N., The place of gold in rapid tests, *IVD Technology*, 2000 (March), p.37.
21. Rocks, B. F., Bertram, V. M. and Bailey, M. P., Detection of antibodies to the human immunodeficiency virus by a silver-enhanced gold-labelled immunosorbent assay, *Ann Clin Biochem*, 27, 114, 1990.
22. Albrecht, R. M. and Meyer, D. A., Size selective synthesis of colloidal platinum nanoparticles for use as high resolution EM labels, *Microsc Microanal*, 8, (Suppl 2: Proceedings), Lyman, C. E. et al., Eds., Cambridge University Press, New York, NY, 124, 2002.

23. Grubisha, D. S., et al., Femtomolar detection of prostate-specific antigen: an immunoassay based on surface-enhanced Raman scattering and immunogold labels, *Anal Chem*, 75, 5936, 2003.
24. Dulkeith, E., et al., Fluorescence quenching of dye molecules near gold nanoparticles: radiative and nonradiative effects, *Phys Rev Lett*, 89, 203002, 2002.
25. Malicka, J., Gryczynski, I. and Lakowicz, J. R., DNA hybridization assays using metal-enhanced fluorescence, *Biochem Biophys Res Commun*, 306, 213, 2003.
26. Moller, R., et al., DNA probes on chip surfaces studied by scanning force microscopy using specific binding of colloidal gold, *Nucleic Acids Res*, 28, E91, 2000.
27. Hamilton, J. F. and Logel, P. G., The minimum size of silver and gold nuclei for silver physical development, *Photogr Sci Eng*, 18, 507, 1974.
28. Hacker, G. W., et al., Silver acetate autometallography: an alternative enhancement technique for immunogold-silver staining (IGSS) and silver amplification of gold, silver, mercury and zinc tissues, *J Histotechnol*, 11, 213, 1988.
29. Hainfeld, J. F. and Powell, R. D., Silver- and gold-based autometallography of Nanogold, in *Gold and Silver Staining: Techniques in Molecular Morphology*, Hacker G.W., and Gu J., Eds, CRC Press, Boca Raton, London, New York & Washington, D.C., 2002, pp. 29-46.
30. Hainfeld, J. F., et al., Gold-based autometallography, *Microsc Microanal*, 5, (Suppl 2: Proceedings), G. W. Bailey, et al., Eds., Springer-Verlag, New York, NY, 1999, 486
31. Hainfeld, J. F. and Powell, R. D., Silver- and gold-based autometallography of Nanogold, in *Gold and Silver Staining: Techniques in Molecular Morphology*, Hacker G.W. and Gu, J., Eds., CRC Press, Boca Raton, London, New York & Washington, D.C., 2002, chap. 3, pp. 29-46.
32. Owen, G. R., et al., Enhancement of immunogold-labelled focal adhesion sites in fibroblasts cultured on metal substrates: problems and solutions, *Cell Biol Int*, 2001, 25, 1251.

33. Powell, R. D. and Hainfeld, J. F., Combined fluorescent and gold probes for microscopic and morphological investigations, in *Gold and Silver Staining: Techniques in Molecular Morphology*, Hacker G. W. and Gu, J., Eds., CRC Press, Boca Raton, London, New York & Washington, D.C., 2002, chap. 7, pp. 107-116.
34. Powell, R. D., et al., A covalent fluorescent-gold immunoprobe: "simultaneous" detection of a pre-mRNA splicing factor by light and electron microscopy, *J Histochem Cytochem*, 1997, 45, 947.
35. Robinson, J.M. and Vandr , D. D., Efficient immunocytochemical labeling of leukocyte mikrotubules with FluoroNanogold: an important tool for correlative microscopy, *J Histochem Cytochem*, 1997, 45, 631.
36. Takizawa, T., Suzuki, K. and Robinson, J. M., Correlative microscopy using FluoroNanogold on ultrathin cryosections. Proof of principle, *J Histochem Cytochem*, 1998, 46, 1097.
37. Powell, R. D., et al., Combined Cy3 / Nanogold conjugates for immunocytochemistry and in situ hybridization, *Microsc Microanal*, 5, (Suppl 2: Proceedings), Bailey, G. W., et al., Eds, Springer-Verlag, New York, NY; 1999, 478.
38. Takizawa, T. and Robinson, J. M., Correlative microscopy of ultrathin cryosections is a powerful tool for placental research, *Placenta*, 2003, 24, 557-565.
39. Takizawa, T., and Robinson, J. M.: Ultrathin cryosections. An important tool for immunofluorescence and correlative microscopy, *J Histochem Cytochem*, 2003, 51, 707.
40. F rster, Th., Zwischenmolekulare Energiewandung und Fluoreszenz, *Ann Physik*, 1948, 2, 55.
41. Powell, R. D., et al., Combined fluorescent and gold immunoprobes: Reagents and methods for correlative light and electron microscopy, *Microsc Res Tech*, 1998, 42, 2.
42. Kandela, I. K., et al., Fluorescence quenching by colloidal heavy metals: Implications for correlative fluorescence and electron microscopy studies, *Microsc Microanal*, 9, (Suppl 2: Proceedings), Piston, D., et al., Eds., Cambridge University Press, New York, NY, 2003, 1194CD.

43. Ribrioux, S., et al., Use of nanogold- and fluorescent-labeled antibody Fv fragments in immunocytochemistry, *J Histochem Cytochem*, 1996, 44, 207.
44. Lackie, P. M., Immunogold silver staining for light microscopy, *Histochem Cell Biol*, 1996, 106, 9.
45. Matsubara, A., et al., Organization of AMPA receptor subunits at a glutamate synapse: A quantitative immunogold analysis of hair cell synapses in the rat organ of Corti, *J Neuroscience*, 1996, 16, 4457.
46. Nusser, Z., et al., Relative densities of synaptic and extrasynaptic GABAA receptors on cerebellar granule cells as determined by a quantitative immunogold method, *J Neuroscience*, 1995, 15, 2948.
47. Yi, H., et al., A novel procedure for pre-embedding double immunogold-silver labeling at the ultrastructural level, *J Histochem Cytochem*, 2001, 49, 279.
48. Mayer, G., et al., Introduction of a novel HRP substrate-nanogold probe for signal amplification in immunocytochemistry. *J Histochem Cytochem*, 2000, 48, 461.
49. Hainfeld, J. F., et al., Enzymatic metallography: a simple new staining method, *Microsc Microanal*, 8, (*Suppl 2: Proceedings*), Voekl, E., et al., Eds., Cambridge University Press, New York, NY, 2002, 916CD.
50. Tubbs R., et al., Enzyme metallography (EnzMet): a novel second generation bright field assay for detection of HER2 gene amplification in breast carcinoma, *J Mol Diagn (AMP 2003 Meeting Abstracts)*, 2003, 5, 279.
51. Furuya, F. R., et al., unpublished results, 2003.
52. Hainfeld, J. F., Furuya, F. R. and Powell, R. D. Metallosomes. *J Struct Biol*, 127, 152-160 (1999).
53. Adler-Moore, J., AmBisome targeting to fungal infections, *Bone Marrow Transplantation*, 1994, 14, S3.
54. Thurston, G., et al., Cationic liposomes target endothelial cells in tumors and chronic inflammation in mice, *J Clin Invest*, 1998, 101, 1401.

55. Hacker, G. W., et al., High-performance Nanogold in situ hybridization and in situ PCR, *Cell Vision*, 1996, 3, 209
56. Hacker, G. W., et al., In situ localization of DNA and RNA sequences: Super-sensitive in situ hybridization using streptavidin-Nanogold-silver staining: Minireview, protocols and possible applications. *Cell Vision*, 1997, 4, 54.
57. Zehbe, I., et al., Sensitive in situ hybridization with catalyzed reporter deposition, streptavidin-Nanogold, and silver acetate autometallography. Detection of single-copy human papillomavirus, *Am J Pathol*, 1997, 150, 1553.
58. Tubbs, R., et al., Gold-facilitated in situ hybridization: a bright-field autometallographic alternative to fluorescence in situ hybridization for detection of HER-2/neu gene amplification, *Am J Pathol*, 2002, 160, 1589.
59. Tubbs, R., et al., Interobserver interpretative reproducibility of GOLDFISH, a first generation gold-facilitated auto-metallographic bright field in situ hybridization assay for HER-2/neu amplification in invasive mammary carcinoma, *Am J Surg Pathol*, 2002, 26, 908.
60. Tanner, M., et al., Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples, *Am J Pathol*, 2000, 157, 1467.
61. Gupta, D., et al., Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer, *Am J Clin Pathol*, 2003, 119, 381.
62. Arnould, L., et al., et al., Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer, *Br J Cancer*, 2003, 88, 1587.
63. Tubbs, R. R., et al., unpublished results, 2003.
64. Hainfeld, J. F., et al., Combined Alexa-488 and nanogold antibody probes, *Microsc Microanal*, 8, (*Suppl 2: Proceedings*), Lyman, C. E., et al., Eds., Cambridge University Press, New York, NY, 2002, 1030CD.

65. Schroeder-Reiter, E., Houben, A. and Wanner, G., Immunogold labeling of chromosomes for scanning electron microscopy: A closer look at phosphorylated histone H3 in mitotic metaphase chromosomes of *Hordeum vulgare*, *Chromosome Research*, 2003, 11, 585.
66. Powell, R. D., et al., Dual-labeled probes for fluorescence and electron microscopy, *Microsc Microanal*, 8, (*Suppl 2: Proceedings*), Bailey, G. W., et al., Eds., Springer, New York, NY, 1998, 992.
67. Rosa-Molinar, E., et al., Colloidal Gold Conjugate of Recombinant Cholera Toxin B-Subunit of Alexa Fluor and Dextran-Texas Red-Nanogold Fluorescent Dyes for Use in Correlative Microscopy and Intravital Imaging, *Microsc Microanal*, 9, (*Suppl 2: Proceedings*), Piston, D., et al., Eds., Cambridge University Press, New York, NY, 2003, 182.

## FIGURE LEGENDS

1. Mechanism of silver enhancement reaction, showing half-cell potentials.
2. (a) and (b) Intraductal comedo-carcinoma of the breast, stained for HER2 using polyclonal c-erb-B2 primary antibody, followed by EnVision secondary polymer-antibody-HRP conjugate, developed with (a) DAB (H and E counterstain) or (b) enzyme metallography (methyl green counterstain) (bar = 10  $\mu$ m); (c) HER2 *in situ* hybridization in non-amplified tissue, using Zymed polymer-antibody reagents with hematoxylin counterstain (400 X original magnification); (d) Her-2/neu in highly amplified tissue, using Zymed polymer-antibody reagents with hematoxylin counterstain (400 X original magnification).
3. Structure of Nanogold-conjugated lipids.

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