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3 Silver- and Gold-Based Autometallography of Nanogold®

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INTRODUCTION

For many applications, silver salt-based autometallography (often also called silver enhancement or silver development)[5,8] is required to visualize colloidal gold (1–5 nm in diameter) or the small 1.4 nm Nanogold® particles (Nanoprobes, Yaphank, NY, USA).[11] Although even Nanogold may be seen directly by scanning–transmission electron microscopy (STEM), by transmission EM (TEM; in thin sections without stain or ice-embedded cryo-EM samples), energy filtered TEM, and scanning EM (SEM), silver enhancement makes viewing in the EM more facile since the particles are enlarged to approximately 10 to 20 nm, convenient for most specimens. Autometallographic (AMG) enhancement is required in order to visualize smaller gold particles such as Nanogold for light microscopy (LM) or in blots or gels. This chapter includes the following protocols:

- Protocol for HQ silver enhancement of Nanogold.
- Protocols for use of silver-enhanced Nanogold with osmium tetroxide.
  A: Procedure using reduced concentration of OsO_4.
  B: Procedures for gold toning.
- Protocol for HQ silver enhancement of Nanogold in pre-embedding immunocytochemistry for cell cultures.
- Protocol for gold enhancement of Nanogold for EM.
- Protocol for gold enhancement of Nanogold for LM.
- Protocol for staining blots with Nanogold and silver enhancement.
- Protocol for staining gels with Nanogold and silver enhancement.

Commonly used heavy metal stains such as osmium tetroxide and lead citrate usually obscure the 1.4 nm gold particles, unless they have been so enhanced. The enhancement process generally follows immunolabeling with Nanogold-labeled Fab′ fragments, Nanogold-labeled IgG, or Nanogold-labeled streptavidin, and can be applied to pre-embedding, postembedding, or ultrathin cryosection protocols. Examples of the development of Nanogold for EM are shown in Figures 3.1 and 3.2. Enhancement is essentially a simple procedure in which the EM grid is simply floated on a drop of developer for several min For LM, silver enhancement is generally always required, and slides may be covered with the developer after immunolabeling with the gold antibody. Development times are

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generally 5 to 10 min longer than those required for EM.

A new procedure that deposits gold instead of silver is now available.[12] This has the advantages of lower background in some cases, higher electron density, which gives higher contrast for EM viewing, a much better backscatter signal for SEM, and full compatibility with OsO_4, which can dissolve or etch silver.[3] Protocols are given for these enhancement procedures.

Silver or gold enhancement can also be used to enhance the signal from Nanogold probes to the point where they are visible with the naked eye. This renders gold labeling visible on gels and blots. This is useful in molecular biology where gels are run, and where it can be used to distinguish bands containing gold-labeled proteins from those that do not; for example, one lane can be stained with Coomassie® blue for protein, and the another with AMG, which will show only those bands that are gold labeled.[7,24] Dot blots are very useful for checking the metal enhancement process and can be used to determine development times for EM.[4] They are also used to quickly assay or troubleshoot an antigen labeling experiment. In a typical dot blot, the target antigen is placed in dilutions on nitrocellulose; subsequent incubations with primary and secondary (Nanogold-labeled) antibodies, followed by AMG, reveal the sensitivity of antigen detection and provide a format in which dilutions of primary and secondary antibodies or other parameters can be varied to optimize antigen labeling.[10,16] Therefore, we include protocols for use with gels and blots.

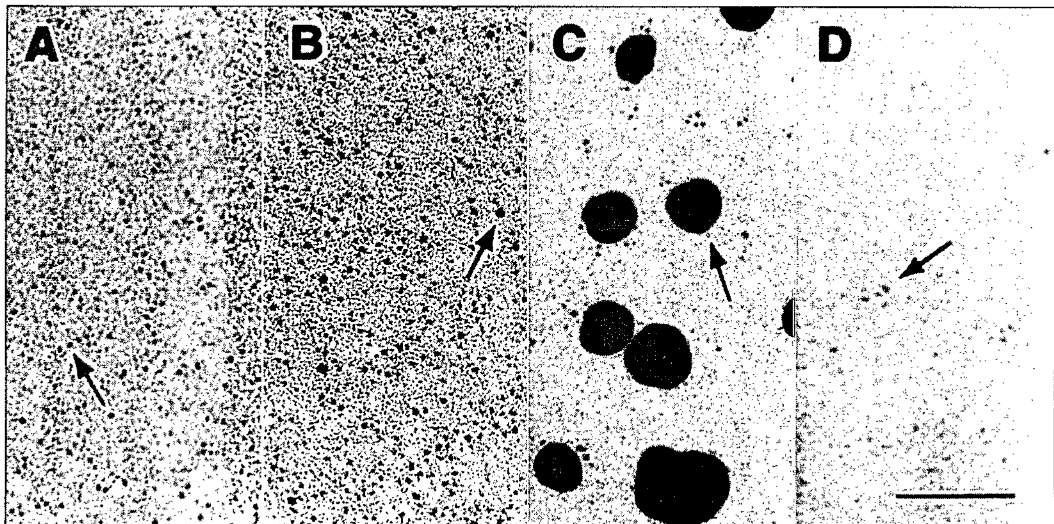

Figure 3.1. Silver enhancement of Nanogold clusters. (A) TEM photomicrograph of Nanogold clusters without enhancement. Arrow points to a 1.4 nm gold particle. (B) Nanogold clusters after 30-sec development (IntenSE™ M; Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) giving 1.7 to 3.3 nm particles. Arrow shows one that is 2.9 nm. (C) Nanogold (more dilute) after 3-min silver development, showing 11 to 40 nm particles. Arrow points to a 19 nm silver grain. (D) Control with no Nanogold but exposure to 3 min of development, showing minimal background spots (arrow). Bar = 0.040 µm. (Reprinted with permission from Hainfield, J.E. and F.R. Furuya. 1992. A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J. Histochem. Cytochem.* 40: 177–184.)

STAINING PROTOCOLS

Protocol 1. HQ Silver Enhancement of Nanogold

HQ Silver (Nanoprobes) is a commercial silver enhancement kit which is optimized for high ultrastructural preservation and uniform particle size in EM.

Materials and Reagents

- Phosphate-buffered saline (PBS) buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.4.
- PBS-BSA (bovine serum albumin) buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, and 1.0% BSA, fraction V by heat shock (Sigma, St. Louis, MO, USA), pH adjusted to 7.4.
- HQ Silver reagent.
- Deionized or distilled water.

Procedure

1. Rinse with deionized water (2 times for 5 min).
2. Float grid with specimen on freshly mixed developer for 1 to 8 min or as directed in the instructions for the silver reagent. More or less time can be used to control particle size. A series of different development times should be tried, to find the optimum time for your experiment. With HQ Silver, a development time of 4 min gives 15 to 40 nm round particles. Since HQ Silver is light sensitive, it should be handled in a darkened room, using a safelight, or inside a covering box to avoid the generation of nonspecific background.
3. Rinse with deionized water (3 times for 1 min).
4. Mount and stain as usual.

Protocols 2 A–C. Silver Enhancement of Nanogold with Osmium Tetroxide

In some cases, OsO_4 will oxidize the deposited silver back into solution, resulting in loss of signal. One of three procedures is recommended in such cases: (A) use of lower concentrations of OsO_4; (B) gold toning using either procedure 2B or procedure 2C; or (C) use of gold enhancement (discussed later). Investigators therefore have a choice of procedures.

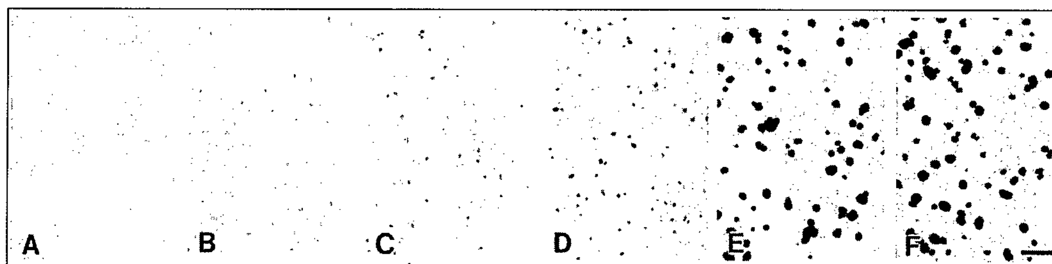

Figure 3.2. Time course for silver enhancement of Nanogold. (A) Gold particles (1.4 nm) adhered to poly-L-lysine-treated formvar-coated EM grid but not incubated with silver enhancement solution. The gold was not visualized by standard transmission EM at this magnification. (B–F) Nanogold particles adhered to grids as in panel A and then incubated with the silver enhancement solution for (B) 1 min, (C) 2 min, (D) 3 min, (E) 4 min, and (F) 5 min. The silver-enhanced gold particles were evident as early as 1 min and continued to increase in size with longer enhancement times. The results of this preparation are typical; however, slight variations in development time were observed with different batches of silver enhancement solution. Bar = 0.1 µm. (Reprinted with permission from Takizawa, T. and J.M. Robinson. 1994. Use of 1.4–nm immunogold particles for immunocytochemistry on ultra-thin cryosections, *J. Histochem. Cytochem.* 42:1615–1623.)

Protocol 2A. Procedure Using Reduced Concentration of OsO_4

This procedure is reported for cells,[23] but may be adapted to tissues.

Materials and Reagents

- PHEM buffer, prepared as follows: 60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, 2 mmol/L $MgSO_4$, pH 6.9.
  Abbreviations used in this buffer system are:
  PIPES = piperazine-N,N′-bis[2-ethanesulfonic acid], can also be written as 1,4-piperazinediethanesulfonic acid
  HEPES = N-[2-hydroxyethyl]piperazine-N′-[4-nutanesulfonic acid]
  EGTA = ethyleneglycol-bis (beta-aminoethyl ether) N,N,N′,N′-tetra-acetic acid
- PBS buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.4.
- PBS+ buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, with 1% normal goat serum, 0.1% saponin, 50 mmol/L glycine, 0.1% fish skin gelatin, 1 mg/mL BSA, and 0.02% NaN_3.
- Glutaraldehyde.
- 50 mmol/L HEPES with 200 mmol/L sucrose, pH 5.8.
- Fixer: 250 mmol/L sodium thiosulfate and 20 mmol/L HEPES, pH 7.4.

Procedure

1. Rinse cells with PHEM buffer, pH 6.9, for 30 sec.
2. Fix cells in 0.7% glutardialdehyde for 15 min in PHEM buffer (use a non-amine containing buffer, i.e., do not use Tris-buffer).
3. Lyse cells for 15 min in PHEM buffer containing 0.5% Triton® X-100.
4. Rinse cells in 3 changes of PBS, pH 7.4, over 15 min.
5. Quench glutaraldehyde with 2 changes of $NaBH_4$ (1 mg/mL in Tris-buffered saline, pH 7.4) over 15 min.
6. Wash cells with 3 changes of PBS with 1% normal goat serum, 0.1% saponin, 50 mmol/L glycine, 0.1% fish skin gelatin, 1 mg/mL BSA, and 0.02% NaN_3 (PBS+).
7. Incubate cells with primary antibody (usually 1:250 dilution or 1:500 dilution of ascites fluid) for 60 min at 37°C.
8. Rinse 3 times in PBS+.
9. Incubate with Nanogold antimouse Fab′ (or IgG) (1:50 dilution) for 60 min at 37°C.
10. Wash 3 times with PBS+.
11. Postfix with 1.6% glutaraldehyde in PBS for 15 min.
12. Wash 4 times with 50 mmol/L HEPES with 200 mmol/L sucrose, pH 5.8, over 30 min.
13. Silver enhance for 5 to 20 min, shielding from light.
14. Rinse 3 times over 5 min in fixer (250 mmol/L sodium thiosulfate and 20 mmol/L HEPES, pH 7.4).
15. Wash 3 times over 15 min with 0.1 mol/L phosphate, pH 7.4, with 0.1 mol/L sucrose.
16. Osmicate with 0.1% OsO_4 for 30 min.
17. Dehydrate and embed; section.
18. Stain thin sections with uranyl acetate and lead citrate.

Note that since silver ions in the silver enhancer precipitate with chloride ions, all PBS and other chloride buffers must first be removed. This is generally done with

water washes, but in the above procedure, a more physiological wash buffer is used (Step 12, HEPES-sucrose).

Protocol 2 B–C. Procedures for Gold Toning

Note: Treatment with osmium tetroxide followed by uranyl acetate staining can lead to much more drastic loss of the silver-enhanced Nanogold particles. This may be prevented by gold toning.

Procedure 2B[2,3]

1. After silver enhancement, wash thoroughly with deionized water.
2. 0.05% gold chloride: 10 min at 4°C.
3. Wash with deionized water.
4. 0.5% oxalic acid: 2 min at room temperature.
5. 1% sodium thiosulfate (freshly made) for 1 h.
6. Wash thoroughly with deionized water and embed according to usual procedure.
7. Now osmium staining may be performed.

Procedure 2C[18]

1. Rinse twice quickly in distilled water.
2. 0.05 mol/L sodium acetate (1 min) then rinse again quickly.
3. 0.05% tetrachloroauric acid (2 min).
4. Rinse thoroughly in distilled water for 10 min, then osmicate.

Protocol 3. HQ Silver Enhancement of Nanogold in Pre-Embedding Immunocytochemistry for Cell Cultures

This procedure has been described by Tanner and coworkers and is reported to give significantly higher densities of silver-enhanced gold particles than other methods.[21] An example of the results is shown in Figure 3.3.

Materials and Reagents

- Sodium phosphate buffer: 0.1 mol/L sodium phosphate, pH adjusted to 7.4.
- PBS buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.4.
- Glutaraldehyde and paraformaldehyde.
- HQ Silver reagent.
- Deionized or distilled water.

Procedure

1. Fix for approximately 45 min (for monolayer cultures) with one of the following: (1) 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4, or (2) 2% paraformaldehyde with 0.05% to 0.1% glutaraldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4.
2. Wash with 0.1 mol/L sodium phosphate buffer, pH 7.4, 3 times for 5 min each.
3. Blocking and permeabilize the cells with PBS with 5% goat serum, 0.1% sodium azide, and 0.1% saponin for 1 h.
4. Incubate with primary antibody made in PBS with 5% normal goat serum, 0.1% saponin, and 0.1% sodium azide for 1 h at room temperature.
5. Wash with PBS with 1% goat serum and 0.1% sodium azide for 3 to 4 times for 5 min.
6. Incubate with Nanogold-labeled Fab′ antirabbit or mouse (depending on the primary antibody) secondary antibody conjugate (4 μL) in 1 mL of PBS with 1% goat serum and 0.1% sodium azide for 1 h at room temperature.
7. Wash with PBS containing 1% goat serum with 0.1% sodium azide once, then with PBS twice.

8. Fix with 2% glutaraldehyde in PBS for 30 min.
9. Wash 3 times in PBS. Store overnight.

Next day:

10. Wash with water thoroughly.
11. Perform silver enhancement (HQ Silver enhancement kit).
13. Wash in water. Check under LM carefully; only process the promising specimens for EM.
14. Wash in 0.1 mol/L phosphate buffer, pH 7.4.
15. 0.2% OsO_4 in 0.1 mol/L phosphate buffer for 30 min.
16. Wash, stain with uranyl acetate, dehydrate in ethanol, and embed.

Protocol 4. Gold Enhancement of Nanogold for EM

Materials and Reagents

- PBS buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.4.
- PBS-BSA buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, and 1.0% BSA, fraction V by heat shock, pH adjusted to 7.4.
- GoldEnhance™ EM reagent (Nanoprobes).

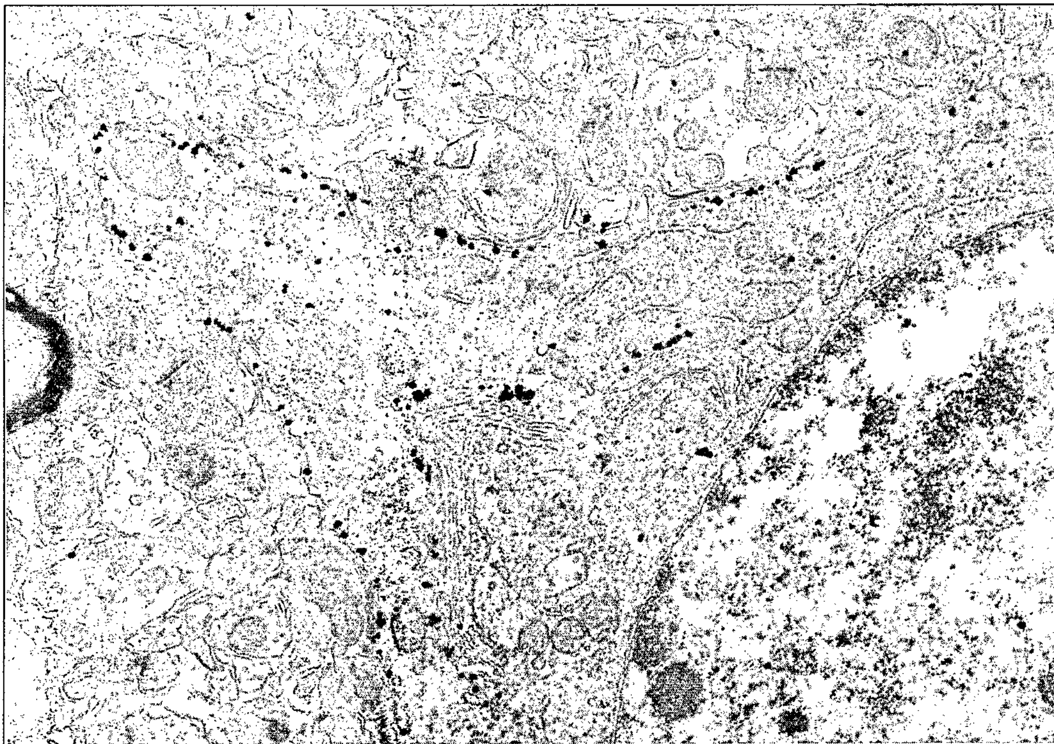

Figure 3.3 EM immunocytochemistry of the K^+ channel, Kv2.1, in brain neurons. The silver-enhanced (HQ Silver) gold grains (Nanogold-anti-mouse Fab′) are distinct on the plasma membrane of the neuronal soma and large dendrites. The plasma membranes facing astrocytic processes shows the heaviest staining, with many more immunograins facing astrocytes than facing synaptic terminals. Intracellularly, the Golgi apparatus is positively stained. Full width, 6.15 μm. (Reprinted with permission from Du, J., et al., 1998. *Neuroscience*, 84:37–48.)

Procedure

1. Incubate with the immunogold or Nanogold conjugate according to your usual or recommended protocol.
2. Optional: Postfix with 1% glutaraldehyde in PBS.
3. Wash 3 times for 5 min with PBS with 50 mmol/L glycine (after glutaraldehyde postfix only—to remove aldehydes).
4. Wash 3 times for 5 min in PBS-BSA.
5. Wash 3 times for 5 min in distilled water.
6. Gold enhancement (GoldEnhance kit): use equal amounts of the four components (Solutions A, B, C, and D); prepare about 40 µL of reagent per grid. A convenient method is to use one drop (approximately 10 µL) from each bottle. After mixing, a drop may be placed on a sheet of parafilm and a grid floated on it for the required time.
   a. First mix Solution A (enhancer: green cap) and Solution B (activator: yellow cap).
   b. Wait 5 min.
   c. Add Solution C (initiator: purple cap), then Solution D (white cap) and mix.
   d. Develop for the optimal particle size (usually between 3–20 min).
7. Rinse with distilled water.

Figure 3.4a shows results obtained using GoldEnhance to enlarge 5 nm cells in tissue sections.[12]

Protocol 5. Gold Enhancement of Nanogold for LM

The following procedure was developed for gold enhancement of *in situ* hybridization (ISH) specimens by Cheung, Hauser-Kronberger, and Hacker, in collaboration with the authors,[12] as a modification of the Nanogold-silver staining procedure;[9] an example of the results obtained using this method is shown in Figure 3.4b. It has been found to be effective for enhancement of tissue sections for LM observation. We have found enhancement duration

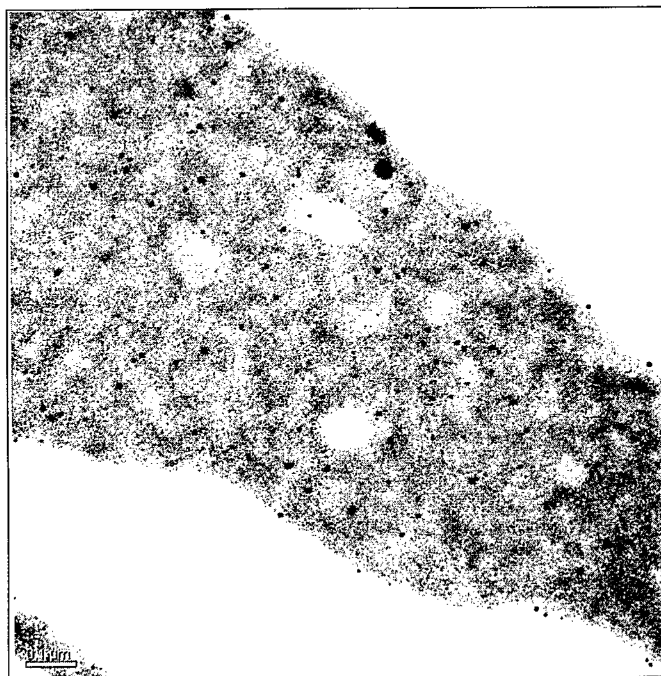

Figure 3.4a Electron micrograph of human testis. (Full width, 1.45 µm). DNA in spermatids was labeled with mouse anti-DNA primary (Roche Molecular Biochemicals, Indianapolis, IN, USA), then biotinylated antimouse antibody (Amersham Pharmacia Biotech), followed by Nanogold–streptavidin, followed by gold autometallography (8 min). (Reprinted with permission from Hainfeld, J.F. et al., 1997. *Proc. 57th Ann. Mtg., Micros. Soc. Amer.*, Springer-Verlag, New York.)

times of 10 to 20 min 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. A similar procedure may be used for blotting applications; a comparison of silver enhancement and GoldEnhance development is shown in Figure 3.4c. You should follow your normal procedure up to the application of the gold conjugate; the protocol below describes the steps after this:

Materials and Reagents

- PBS buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.6.

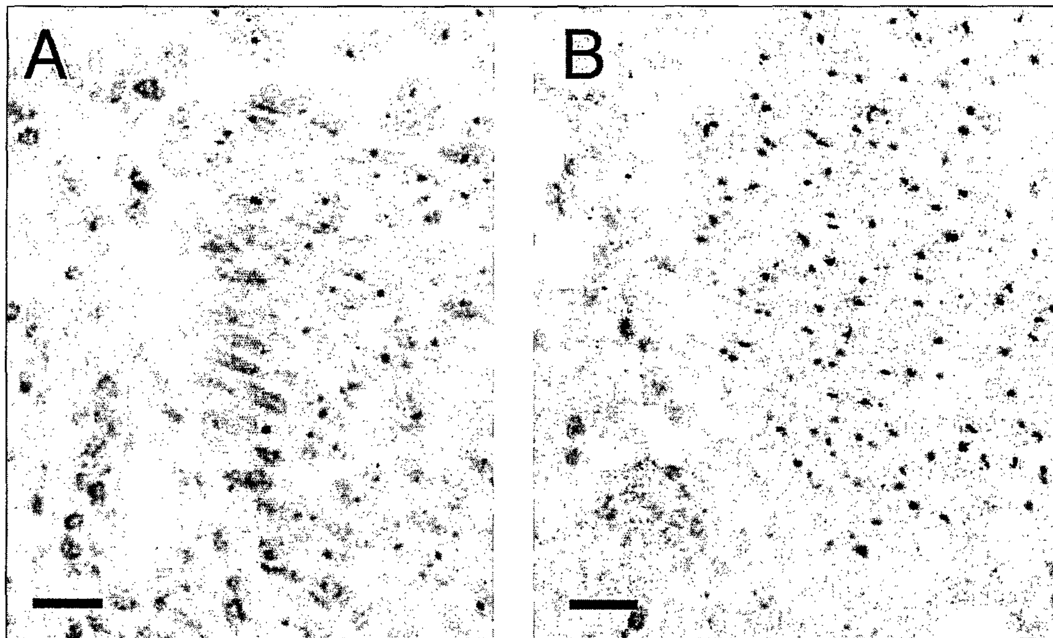

Figure 3.4b. **Human papillomavirus (HPV) 16/18 in cervical carcinoma.** LM photomicrographs of formalin-fixed serial paraffin sections of cervical squamous cell carcinoma, *in situ* hybridized for HPV-16/18 using a biotinylated probe (Pathogene-HPV kit; Enzo Diagnostics, Farmingdale, NY, USA) (Bar = 10 µm). (A) Direct detection using streptavidin-peroxidase. (B) Direct detection using Nanogold–streptavidin followed by gold autometallography for 18 min. (Reprinted with permission from Du, J. et al., *Neuroscience* 84:37–48.)

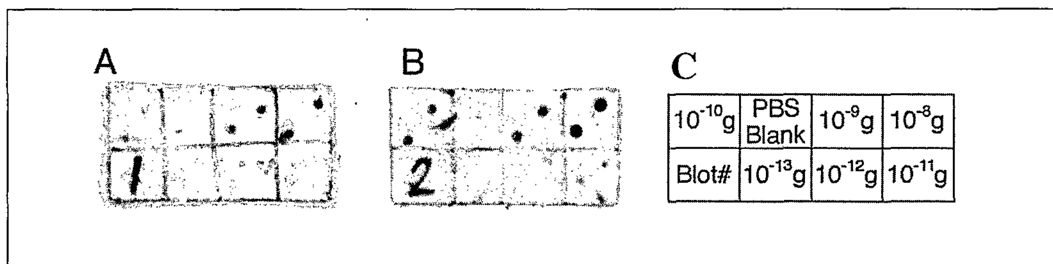

Figure 3.4c. **Immunoblot detection of mouse IgG on nitrocellulose.** Gold-goat antimouse IgG (15 nm) is used and amplified with (A) silver AMG (LI Silver) and (B) gold AMG. (C) Key showing the amounts of mouse IgG in each spot for the corresponding divisions of the blots. (Reprinted with permission from Hainfeld, J.F. et al., 1997. *Proc. 57th Ann. Mtg., Micros. Soc. Amer.*, Springer-Verlag, New York.)

- PBS-gelatin buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, and 0.1% gelatin (high purity), pH adjusted to 7.6.
  Optional: Background may be reduced by using 0.5 mol/L NaCl and 0.05% Tween® 20 in this buffer.
- GoldEnhance LM reagent (Nanoprobes).

Procedure

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 for 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 µL per slide.
   a. Dispense Solution A (enhancer: green cap) into a clean tube or dish, add Solution B (activator: yellow cap), and mix thoroughly.
   b. Wait 5 min.
   c. Add Solution C (initiator: purple cap) and Solution D and mix thoroughly.
   d. Apply 1 to 2 drops (approximately 80 µL, sufficient to cover the specimen) to the slide.
   e. Develop specimen for 10 to 20 min. More or less time can be used to control particle size and intensity of signal.
6. When optimum staining is reached, immediately stop by rinsing carefully with deionized water.

Protocol 6. Staining of Blots with Nanogold and Silver Enhancement

The basic procedure for gold immunoblotting has been described by Moeremans et al.,[15] which may be followed. For best results, the membrane should be hydrated before use by simmering in gently boiling water for 15 min. Best results are obtained when the antigen is applied using a 1-µL

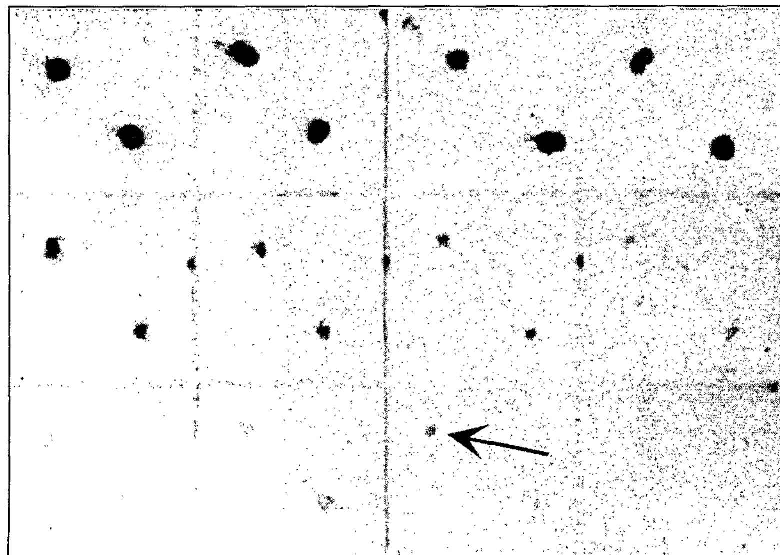

Figure 3.5. Immunoblot of serial dilutions of Mouse IgG. Spotted onto a hydrated nitrocellulose membrane, detected using Nanogold-labeled Fab′ goat antimouse IgG, then developed using LI Silver. The last visible spot (arrow) contains 0.1 pg of the target IgG.

capillary tube (Figure 3.5). The procedure for immunoblots is as follows:[10,16]

Materials and Reagents

- Buffer 1 (Blocking): 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, and 4.0% BSA (fraction V by heat shock), pH adjusted to 7.4.
- Buffer 2 (Incubation): 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, 0.8% BSA (fraction V by heat shock), and 1.0% normal serum from the host animal of the Nanogold conjugate antibody, pH adjusted to 7.4. Optional: Even lower backgrounds may be obtained with 0.5 mol/L NaCl and 0.05% Tween 20.
- Buffer 3 (Wash): 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, 2 mmol/L sodium azide, and 0.8% BSA (fraction V by heat shock), pH adjusted to 7.4.
- Buffer 4 (PBS): PBS buffer: 0.02 mol/L sodium phosphate buffer with 0.15 mol/L sodium chloride, pH adjusted to 7.4.
- Glutaraldehyde.
- 0.05 mol/L disodium EDTA, pH 4.5.
- Silver enhancement reagents, e.g., according to Danscher[5] or to Hacker et al.[8,9]

Procedure

1. Spot 1-μL dilutions of the antigen in Buffer 4 onto hydrated nitrocellulose membrane. Use an antigen concentration range from 100 ng to 0.01 pg/μL.
2. Block with Buffer 1 for 30 min at 37°C.
3. Incubate with primary antibody according to usual procedure (1 or 2 h).
4. Rinse with Buffer 1 (3 times for 10 min).
5. Incubate with a 1/100 to 1/200 dilution of the Nanogold reagent in Buffer 2 for 2 h at room temperature.
6. Rinse with Buffer 3 (3 times for 5 min), then Buffer 4 (2 times for 5 min).
7. Optional (may improve sensitivity): Postfix with glutaraldehyde, 1% in Buffer 4 (10 min).
8. Rinse with deionized water (2 times for 5 min).
9. Optional (may reduce background): Rinse with 0.05 mol/L EDTA at pH 4.5 (5 min).
10. Develop with freshly mixed silver developer for 5 to 25 min as directed in the instructions for the silver enhancement protocol used. Repeating the process for a second time may be beneficial. If performed twice, between the developments, thorough rinsing with deionized or better distilled water is required.

Note: If silver lactate AMG[5] is used, it is advisable to shield preparations from daylight, e.g., within a cupboard. Silver acetate AMG[8,9] is less sensitive to daylight, and development usually can take place under normal laboratory light conditions if not performed for a longer time. If precipitation takes place (solution turns to gray or black), this may be understood as a sign of too much light intensity (in this case, place a dark dustbin on the vials to shield them from daylight). If the solution turns whitish, the quality of the distilled or deionized water is too low, and chloride ions may be present.

11. Rinse several times and thoroughly with deionized water.

Caution: Nanogold particles degrade upon exposure to concentrated thiols

such as beta-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mmol/L and exposure restricted to 10 min or less.

Protocol 7. Staining Gels with Nanogold and Silver Enhancement

Procedure[7,24]

1. After labeling with Nanogold, remove unbound gold particles by column chromatography, sucrose gradient or other purification means. Leaving excess free Nanogold in the sample will interfere with the intended gel staining.
2. Run gel as usual; however, Nanogold is degraded by beta-mercaptoethanol [or dithiothreitol (DTT)], so the sample must not be mixed with a reducing agent, i.e., a nonreducing gel must be run. Normal concentrations of other ingredients [sodium dodecyl sulfate (SDS), etc.] are acceptable.
3. Gel may be electrotransferred to nitrocellulose if desired, although this is not necessary.
4. Rinse gel with several changes of deionized water. Since the silver developer is precipitated by halides, traces of NaCl must be removed.
5. Place the gel or blot in a suitable dish and apply enough freshly prepared LI Silver (Cat. No. 2013; Nanoprobes) to cover the gel. LI Silver is prepared by mixing equal amounts of a and b components. Do not use the usual gel silver stains, which are quite different from LI Silver and do not develop the Nanogold effectively.
6. Watch development of band(s) which should appear brown-black. Aggregates with gold that did not enter the gel or small amounts of free gold may give background staining. Usual development time is 1 to 5 min. Extensive development time (>30 min) will lead to some nonspecific background self-nucleation staining by the developer alone.
7. When optimal staining is reached, stop development by rinsing in deionized water. The final stained gel is now a permanent record.
8. For comparison and visualization of all bands, run a duplicate gel and stain with Coomassie blue or gel silver stain.
9. A Nanogold-labeled molecule may run approximately 15,000 MW higher on the gel due to the added weight of the Nanogold particle (approximately 15,000). However, due to the small hydrodynamic size of the gold cluster, some labeled proteins run close to their native position.

Some results from different gel staining experiments run using different conditions are shown in Figures 3.6a[7] and 3.6b.[11]

TECHNICAL HINTS AND DISCUSSION

AMG is a versatile method with an increasing variety of refinements, which may be applied to a wide variety of specimens. When correctly optimized, Nanogold labeling with silver or gold enhancement can give higher detection sensitivities than competing technologies, such as enzyme-linked detection.[9,25] The results are affected by many factors, and a variety of modifications to these protocols are available that can be used to optimize them for specific systems or experiments or correct problems that may be encountered with the general protocols.

Silver enhancers tend to be divided into two types. The first is often based on silver lactate, which includes a thickening agent or protective colloid, usually gum Arabic, although gelatin and polyethylene glycol (carbowax) have also been used, and is

light sensitive. Examples include the Danscher formulation[7] and the N-propyl-gallate formulation developed by Burry.[4] These may consist of three or more components, and are usually preferred for EM because they produce enhanced particles of a more uniform size and shape and allow improved preservation of ultrastructural morphology. The second type is usually not highly light sensitive, although strong illumination does have an effect, and the formulation is often based on silver acetate, although other silver salts have been used. Examples include the silver acetate AMG solution suggested by Hacker et al.[8] These are simpler to use, usually consisting of two components that are mixed immediately before development, and are preferred for LM and blotting because development can be visually monitored. Krenács and Krenács have reported excellent results with a light insensitive silver acetate developer for post-embedding, which gave very uniform 10 nm spheres from Nanogold at the EM level.[13] Use of a safelight is recommended for these developers, but development

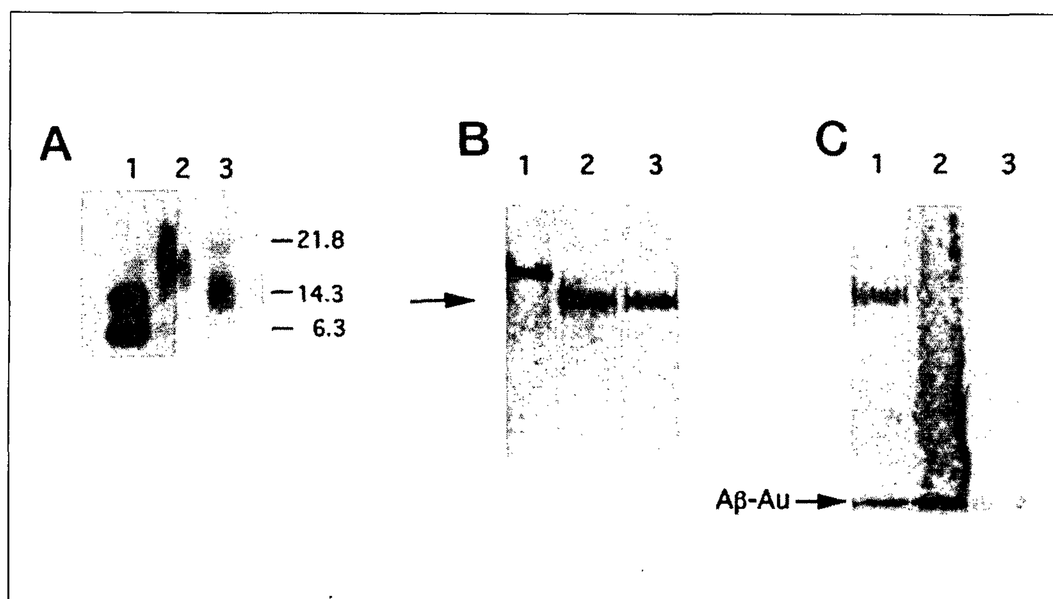

Figure 3.6a. Electrophoretic analysis of proteasome-amyloid β protein (Aβ)-Nanogold complex. (A) Covalent and select conjugation of monomaleimido-Nanogold to proteins requires the presence of a cysteine residue on the protein. Because Aβ lacks cysteines, we used a peptide variant in which the last amino acid (Val^{40}) was substituted with a cysteine residue ($A\beta_{1\text{-}39C40}$). $A\beta_{1\text{-}39C40}$ was coupled to Nanogold as described in Reference 7 to form $A\beta^{Au}$ in which each labeled Aβ molecule was linked to a single gold particle. The product (0.1 μg) was analyzed by 14% Tris-Tricine polyacrylamide gel electrophoresis (PAGE) (lane 2), $A\beta_{1\text{-}39C40}$ (lane 1), and Nanogold (lane 3) were used as controls. Proteins were transferred onto the polyvinylidine fluoride (PVDF) membrane for 30 min at 150 mA at 4°C, and $A\beta^{Au}$ was immunostained with anti-Aβ antibodies (left panel) or stained with the silver enhancement method (right panel). Both staining methods reacted with the same band indicating that $A\beta^{Au}$ migrates as a complex of 17 kDa. Molecular size markers are shown are shown on the right. Note that because gel electrophoresis was performed under denaturing, but not reducing, conditions to prevent thiol degradation of the gold particle, the control lane with the peptide alone shows both the monomer and dimer forms of $A\beta_{1\text{-}39C40}$ (lane 1). (B and C) Electrophoretic characterization of the proteasome-$A\beta^{Au}$ complex. For STEM analysis, the complexes were cross-linked as described in Reference 7. Cross-linked proteasomes (panel B, lane 2) and cross-linked proteasome-$A\beta^{Au}$ complexes (panel B, lane 3) migrated faster than noncross-linked proteasomes (panel B, lane 1). $A\beta^{Au}$ was incubated with proteasome to form proteasome-$A\beta^{Au}$ complex. The complex was detected by Coomassie blue (B) and silver enhancement staining (C). Both staining methods identified the same band confirming the formation of the proteasome-$A\beta^{Au}$ complex. (B) Lane 1, 3 μg of noncross-linked proteasome; lane 2, 3 μg of cross-linked proteasome; lane 3, cross-linked proteasome-$A\beta^{Au}$ complex. (C) Lane 1, cross-linked proteasome-$A\beta^{Au}$ complex; lane 2, 1 μg of cross-linked $A\beta^{Au}$; lane 3, 3 μg of cross-linked proteasome to Nanogold. (Reprinted with permission from Gregori, L., et al., 1997. *J. Biol. Chem.*, 272: 58–62.)

under a box to exclude direct light in a normally lit room is acceptable.

Gold salt-based enhancement is a new procedure, developed by Nanoprobes, in which gold rather than silver is deposited onto gold seed particles.[1,12] This procedure has a number of advantages over silver enhancement. In addition to higher contrast in the electron microscope, greatly increased backscatter signal (for SEM), and resistance to osmium etching, gold enhancement gives a longer time between full development and autonucleation. This means that gold enhancement is more suited to systems requiring extensive washing, or automated processes with longer wait times between steps. Unlike silver, gold is not precipitated by chloride, and therefore gold enhancement can be conducted in the presence of physiological buffers containing saline. Compared with silver enhancement, lower backgrounds have been reported for ISH experiments using Nanogold with gold enhancement as the detection system.[12]

The biggest challenge with AMG is to select the right development time for the desired particle size or staining level. In the light microscope, a slide can be periodically monitored; but for a light sensitive

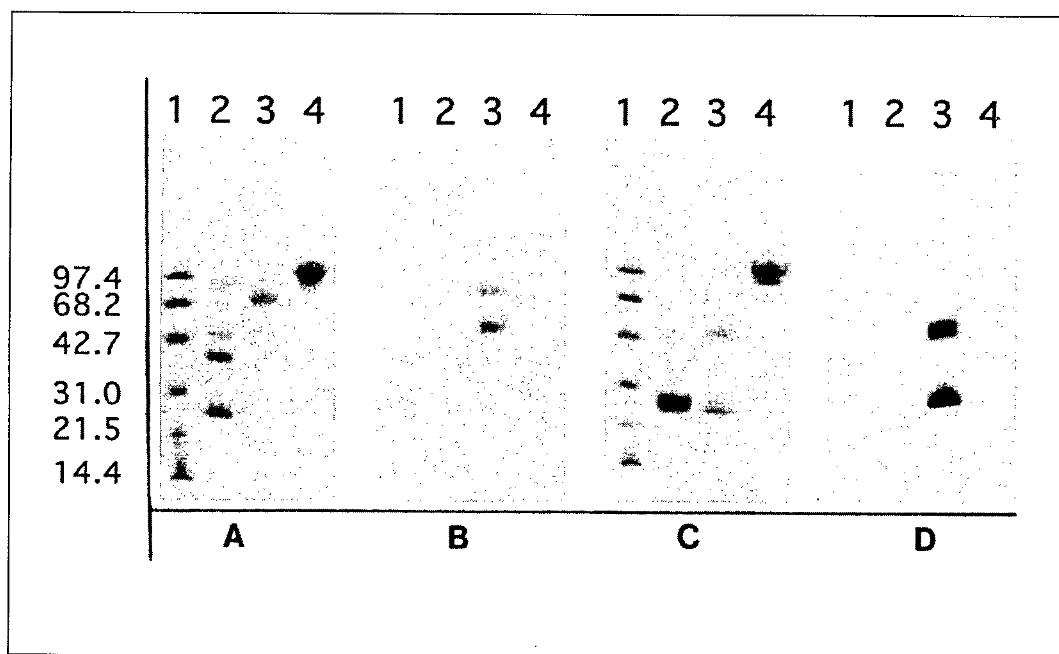

Figure 3.6b. SDS polyacrylamide Phast gels of native and Nanogold-labeled proteins, with development by Coomassie blue or silver-enhancement. Lane 1 is a protein molecular weight standard (values listed on left are in kDa), lane 2 is a native Fab′, lane 3 is a Nanogold-Fab′, and lane 4 is F(ab′)2. Gels A and C are developed with Coomassie blue and gels B and D are developed with a silver enhancer (LI Silver). A and B are gels of samples that were not heated before running, and C and D are gels of samples heated to 100°C in 1.3% SDS for 5 min before running. Gels A and B were identical except for staining, as were gels C and D. The unheated samples show native and Nanogold-labeled Fab′ to run anomalously, showing bands greater than 50 kDa, whereas F(ab′)2 runs at approximately 100 kDa as expected. After heating (gels C and D), the Fab′ runs as expected showing bands at 50 kDa and the single light or heavy chains at 25 kDa. The Nanogold-labeled Fab′ bands are nearly indistinguishable from the native Fab′ bands in this case (gel C, lanes 2 and 3). In all cases, the silver enhancement specifically developed the Nanogold labeled proteins selectively (gels B and D), and unlabeled proteins did not develop (gels B and D, lanes 1, 2, and 4). In addition, Nanogold bands with silver enhancement were intense in less than 5 min, whereas Coomassie staining took 1 h (followed by 1 h of destaining). (Reprinted with permission from Hainfeld, J.F. and F. R. Furuya, 1995. *Immunogold–Silver Staining: Principles Methods, and Applications*, CRC Press, Boca Raton, pp 71–96.)

developer for EM, this is more difficult. Burry has devised a simple test strip method for Nanogold to standardize results from week to week.[4] Nanogold-Fab′ was spotted (approximately 0.5 μL) onto a strip of nitrocellulose at 1:10, 1:50, 1:100, and 1:500 dilutions. The strip was run at the same time as the tissue, and the spots turned faint and then dark brown during development. Particles (15–20 nm) in the TEM corresponded to a medium brown spot at the 1:50 dilution; this time point also was just before silver staining could be perceived in the light microscope.

Several size distribution studies have been reported for silver-enhanced Nanogold. Burry et al.[4a] used N-propyl gallate (NPG) developer over a 1 to 15 min time period to study the enhancement of Nanogold and 1 nm colloidal gold. A linear increase in particle density was found for 1 nm colloidal gold, whereas a sigmoidal curve was observed for Nanogold. However, the size distribution variation (standard deviation) at any particular time point was significantly less for Nanogold.[4] Cultured cell immunolabeling with Nanogold and silver amplification produced good results at 15 min intensification time for LM, but labeling was optimal for EM after a 6 min development, giving an average size of 20 nm particles (10 min gave usable 35 nm particles). Fixed tissue sections required longer silver amplification times (20–25 min) than cultured cells to produce good results, presumably due to the increased time required for the developer to diffuse into the specimens.

Another study documented the size of Nanogold particles adsorbed to poly-L-lysine coated formvar grids, enlarged using the same NPG developer.[20] Particles (10 nm) were obtained after about 3 min, and 25 nm particles were obtained after 5 min. These authors also used this as a quick test (using the EM) to determine optimal development time for each batch of their silver enhancement solution. Nanogold was compared with undecagold and colloidal gold in a third study.[11] Silver-enhanced Nanogold was found to be more sensitive for visual detection of a target antigen than either undecagold or 1 or 3 nm colloidal gold. We typically find silver-amplified immunodot blots using Nanogold conjugates to be 10 to 100 times more sensitive than colloidal gold conjugates (e.g., 10 nm).

Components which improve the performance of silver enhancement reagents include natural products such as gum Arabic, which can vary in composition from lot to lot. Therefore, when using such reagents, it is advisable to test them before using a new batch to ensure that results are reproducible. Tanner and coworkers have used such reagents extensively and, for optimum and consistent performance, recommend the following procedures:[21]

1. Prepare or order sufficient reagent for several experiments (for consistency). Freeze the component solutions in small lots and thaw when needed.
2. Test on grid before use to obtain an approximate reaction time for the required silver particle size. Make up a 1:10 dilution of the Nanogold, place a formvar-coated grid on a drop of this solution, remove excess, and let dry. Then silver enhance the grid. This provides a test of both the potency of the Nanogold (i.e., the proportion of particles which nucleate enhancement), as well as the reaction time and quality of the silver enhancer.
3. The silver enhancement solutions should not be freeze-thawed more than once. Also, storage in the refrigerator is not recommended, since the properties can change with storage time.
4. When making up the silver enhancement solution, if using HQ Silver, pour the most viscous solution (moderator,

Solution B) first into a tube with volume markings. Then add equal volume of Solution A (initiator). Mix the two very well, then add Solution C (activator). Mixing should be both very thorough and very quick. The performance of the HQ Silver can change if it is not used immediately after mixing. Best results are obtained when the reagents are mixed and used quickly.

5. The silver reaction can still change even after thorough water wash. Therefore, strong light should be avoided after silver enhancement.
6. Use a low concentration of OsO_4 (0.2%). The susceptibility of the deposited silver to osmium etching can vary from batch to batch of silver enhancement reagent.

In some experimental systems, background staining — the presence of silver-enhanced particles in areas of the specimen known not to contain the target — can be a problem. This can arise from a number of sources: (1) from unbound Nanogold particles, (2) from unbound primary antibody or probe, or (3) from autonucleation of the silver enhancer solution in the absence of gold particles. Reducing the concentration of the primary antibody or probe or the Nanogold conjugate can reduce or eliminate this problem, as can more extensive washing procedures. Incorporation of a detergent such as Tween 20 or saponin into the procedure can also act to facilitate removal of unbound probe.

We have found that background signal may be reduced or avoided by washing thoroughly with sodium citrate buffer before enhancement.[16] Where HQ Silver is used, 0.02 mol/L sodium citrate buffer at pH 7.0 has been found to be most effective. In preparations utilizing the Danscher silver enhancement protocol,[6] 0.02 mol/L sodium citrate buffer, adjusted to pH 3.5, was most effective. In blots, we find that rinsing with 0.05 mol/L disodium EDTA, pH 4.5, immediately before silver enhancement can reduce background. We attribute this effect to the chelation and removal by the EDTA of transition metal ions, which can act as nucleation sites for silver enhancement.

In addition to the sodium citrate buffer and using a lower concentration of the Nanogold probe, a number of methods have been described for stopping the silver enhancement reaction, or for "back-developing," to remove extraneous deposited silver. These prevent the continuation of the reaction in the specimens after development is complete (for example, if the silver is only slowly removed from the tissue), and may help reduce background signal.

Sodium thiosulfate (1% aqueous solution, freshly made) is a good stop reagent for both silver and gold enhancement and may be used to stop gold or silver development in situations where repeated water washes are insufficient. Washing with deionized water, then incubation with sodium thiosulfate for 1 to 2 min, followed by rinsing thoroughly again with deionized water is usually sufficient to stop development.[22] However, caution should be exercised with this procedure when using gold enhancement. In some experiments, treatment with sodium thiosulfate has been found to reduce signal.

Note: In our experience, it is advisable to avoid stopping the enhancement process by sodium thiosulfate or photographic fixer when using Nanogold for supersensitive DNA or RNA detection. We have often observed a strong reduction of staining when using the stop-bath for more than 1 sec, and one had to be very fast. Instead, but with the risk of obtaining some degree of background staining, thorough washing in distilled water can replace the immediate interruption of the

enhancement process with sodium thiosulfate–photographic fixer by this slower, but less invasive water wash.

Other methods for stopping the AMG reaction include:

1. 1% acetic acid.[19]
2. 1% acetic acid followed by photographic fixer (Agefix; Agfa-Gevaert, or Ilfospeed 200; Ilford Photo, Paramus, NJ, USA).[19]
3. Direct photo fix, using the same photographic fixers listed above.[4]
4. Brief rinse in 2.5% sodium chloride.[19]
5. 15% to 25% aqueous sodium thiosulfate plus 15% sodium sulfite.[5]
6. 1% acetic acid, washes in acetate buffer, toning in 0.05% $HAuCl_4$ for 3 to 10 min, with excess silver removed with 3% sodium thiosulfate.[20] We found that Nanogold-labeled proteins run on a polyacrylamide gel kept low backgrounds when stopped with 10% acetic acid with 10% glucose in water, as opposed to just a water stop.
7. Although not reported for Nanogold labeling, silver overdevelopment of immunogold probes has been used, followed by reversal, to lower the background.[5] A modified Farmer's solution was used for the reversal (0.3 mL 7.5% potassium ferricyanide, 1.2 mL of 20% sodium thiosulfate, 60 mL water) [Reference 4; already reported by Häcker in Springall et al. (19a)]. If the higher concentrations of probe required for fluorescence microscopy continue to result in nonspecific signals after AMG, treatment with this solution after AMG may help to reduce it.

Conversely, in some procedures, little or no development has been found upon AMG. Results may be improved in these systems by changing from commercial silver enhancement reagents to freshly-prepared Danscher and Hacker formulations[5,8,9] or by substituting formaldehyde for glutaraldehyde in postfixation.

Nanogold with silver enhancement may be followed by standard immunocolloidal gold to a different antigen for double labeling. This was achieved by Takizawa and Robinson,[20] who showed that the labels were very distinctly recognizable and that the silver enhancement was gentle enough to preserve antigenicity when the next immunolabel (a 10 nm colloidal gold) was applied. This is useful when one antigen is sparse, since Nanogold generally gives much more dense labeling than colloidal gold.

Nanogold with AMG can also be used in conjunction with other staining procedures for multiple antigen staining. In the electron microscope, the particles are easily distinguished from other stains, and in the light microscope, the black staining is also readily distinguished from other commonly used stains. Two studies have described the use of AMG-enhanced Nanogold in conjunction with enzymatic labeling to distinguish different antigens.[14,17] Nanogold and silver enhancement should be completed before the application of the enzymatic probe. If the enzymatic probe is applied first, the substrate can act as a nucleating agent during AMG enhancement and give nonspecific background staining.

Further optimization of both the formulation and applications of silver and gold enhancement with Nanogold are planned. AMG-enhanced Nanogold offers a unique combination of high spatial resolution and punctate staining for the electron microscope, and the highest sensitivity for LM and blotting. The development of gold enhancement and related technologies makes this process readily applicable to automated staining instruments and molecular diagnostics.

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