5 nm NANOGOLD®

Nanoprobes

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PRODUCT INFORMATION

5 nm AMINO NANOGOLD® LABELING REAGENT

Product Name:	**5 nm AMINO NANOGOLD® (5 nm Positively Charged-Nanogold®)**
Quantity:	**5 nmol**
Catalog Number:	**2221-5NMOL**
Appearance:	**Red solid**
Revision:	**1.0 (December 2024)**

Congratulations on your acquisition of a revolutionary new gold labeling reagent: **5 nm Amino-Nanogold®**. This reagent may be used to label antibodies, proteins, peptides, modified nucleic acids, or any other biomolecule containing an accessible primary amino-group, with 5 nm Nanogold®. Nanogold® is conjugated through discrete, covalent chemical reactions. Unlike conventional colloidal gold which is non-specifically adsorbed to antibodies and proteins, Nanogold® is selectively conjugated at specific sites.[1] Nanogold® does not require additional macromolecules such as BSA for stabilization, so probes are smaller and the gold label can get closer to the site of interest. Conjugates prepared with this reagent have several advantages over colloidal gold conjugates (see below).

Contents

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Non-radioactive and non-carcinogenic.

Product Information

5 nm Amino Nanogold® is a recently developed, highly monodisperse gold nanoparticle label that is stabilized by a layer of coordinated organic ligands. These confer high stability, biocompatibility and water-solubility. These coordinated ligands also incorporate reactive amino groups, These have a specific reactivity towards aldehyde groups, with which they form Schiff bases that may then be reduced to amines. This procedure may be used to label carbohydrate moities in glycoproteins, as shown in Figure 1. It may also be used to label carboxylic acids, or with cross-linking reagents to label other groups, such as primary amines.

Some labeling applications of 5 nm Amino Nanogold®:

- Proteins, which may be labeled at activated *C*-terminal or side-chain carboxyls
- Antibodies, which may be labeled at glycosylated Fc sites.
- Glycoproteins
- Nucleic acids (oligonucleotides, DNA) and peptide nucleic acids (PNA).
- Glycolipids
- Modified sugars or glycosylated small molecules
- Surfaces
- Components of self-assembling nanostructured materials

This reagent as supplied has been lyophilized from methanol and is in the form of a sticky, dark red solid. This should be reconstituted before use in reaction buffer. Nanogold® conjugates can be used in immunoblotting, light microscopy, and electron microscopy to provide clear visibility. They are stable to wide ranges of pH and ionic strength and are not radioactive or carcinogenic.

5 nm Amino Nanogold® should be stored at -20°C.

5 nmol of reagent is supplied, sufficient to ensure complete labeling of up to 5 nmol of amine sites. This corresponds to about 0.5 mg of a protein with a molecular weight of 100,000 with one site labeled.

Nanogold®-labeled biomolecules prepared with 5 nm Amino Nanogold® can be detected or localized by electron microscopy, or light microscopy, as well as on gels and blots, using silver or gold enhancement: Nanogold® will nucleate silver or gold metal deposition, resulting in a dense particle up to 100 nm in size or larger depending on development time and producing a black, brown or purple stain.[3]

Nanoprobes offers a number of silver enhancement and gold enhancement reagents. Detailed instructions may be found with each:

Silver enhancement kits:

2012-45 mL HQ Silver™
Best for EM: Uniform enhancement, low background and excellent ultrastructural preservation.

2013-250 mL LI Silver™
Use to stain Nanogold® labeled proteins or nucleic acids for light microscopic observation, in gels and on blots.

For more information, visit our website:
https://www.nanoprobes.com/products/Silver-Enhancers.html

GoldEnhance™ gold enhancement kits:

2112-28 mL GoldEnhance™ LM
Brown colored stains. High sensitivity and low background.

2113-8 mL GoldEnhance™ EM
High sensitivity and rapid enhancement.

2114-8 mL GoldEnhance™ EM Plus

Uniform enhancement and high sensitivity.

2115-48 mL GoldEnhance™ Blots
Purple colored stain. High sensitivity and rapid enhancement for direct optical and visual detection.

For more information, visit our website:
https://www.nanoprobes.com/products/GoldEnhance.html

Thiol Caution

5 nm Nanogold® particles may be degraded upon prolonged exposure to thiols such as ß-mercaptoethanol or dithiothreitol. If these are required, we recommend that concentrations be kept below 10 mM and exposure times limited to one hour or less. Elevated temperatures may increase the risk of thiol degradation. If possible, monitor reaction mixtures or preparations containing thiols by UV/visible spectroscopy for any change in color, spectral profile, or reduction in absorption at the plasmon resonance peak (close to 520 nm), and remove thiols if any changes are observed.

We recommend that thiol compounds used in the reduction of proteins (or other biomolecules) be separated from reduced protein by gel filtration liquid chromatography before Nanogold® conjugation. Best results are usually obtained using a desalting gel with an appropriate molecular weight cut-off (MWCO) such as one of the Sephadex gels (Cytiva) or 7K MWCO Zeba spin desalting columns, plates or cartridges (Thermo Fisher Scientific). Dialysis is not recommended as it frequently does not afford complete thiol removal in this application. **Do not store 5 nm Nanogold® or its conjugates in the presence of thiols**.

Other Cautions

Nanogold® is stable at temperatures up to 100°C at pH values from 5 to 10 and ionic strengths under 0.3 M, which includes many commonly used or standard biological buffers. However, under demanding conditions, including pH values lower than 4 or ionic strengths above 0.3 M, Nanogold® reagents, labeled specimens or conjugates may be less stable above 50°C, and best results are obtained at room temperature or 4°C. If your experiment requires such conditions, avoid 37°C incubations, and use low temperature embedding media (e.g., Lowicryl) for pre-embedding immunolabeling.[4]

Introduction to Labeling with 5 nm Nanogold®

General considerations

There are important differences between our 5 nm Nanogold® reagents and our smaller, 1.4 nm Nanogold® reagents, which can affect conjugation. Before starting a labeling reaction, you should consider the following:

(a) What is your desired degree of labeling? What ratio of 5 nm Nanogold® : conjugate biomolecule is preferred or acceptable?
(b) Do you require the complete removal of (a) unconjugated 5 nm Nanogold®, and / or (b) unlabeled conjugate biomolecule?
(c) How will you separate the 5 nm Nanogold® conjugate from unconjugated 5 nm Nanogold® and unlabeled conjugate biomolecule?

5 nm Nanogold® has two important differences from the smaller 1.4 nm Nanogold® reagents that may require changes in your method:

(**1**) **5 nm Nanogold® reagents are large molecules**.

In addition to the 5 nm diameter gold core, 5 nm Nanogold® reagents contain a coating of organic molecules, which forms a 'shell' about 2.5 nm thick. Their overall diameter, including this layer, is about 10 nm. This is similar in size or larger than many proteins and biomolecules that are commonly used as probes. For example, IgG, which has a molecular weight (MW) of 150,000 Da, is about 12 nm in length, and each component fragment (Fab, Fab' or Fc) is about 6 nm in length. Based upon an average protein density of 1.35 g/cm^3, a globular protein with the same dimensions as 5 nm Nanogold®, a 10 nm diameter sphere, would have a molecular weight of 4.26 x 10^5 Da (426 kDa). Thyroglobulin, which is used as a high molecular weight standard for chromatographic separations, has a molecular weight of 669,000 Da, and occupies a volume about 1.5 times that of 5 nm Nanogold®. Because proteins usually are not spherically symmetrical, it is reasonable to assume that for separation planning, a 200 kDa protein is about the same size as 5 nm Nanogold®.

This is an important consideration in choosing a method for separating 5 nm Nanogold® conjugates. If you plan to separate conjugates by a size-dependent method such as centrifuge filtration or gel filtration, the best strategy is to use an excess of the smaller of the two reagents, because it is easier to separate unreacted excess smaller reagent. The larger reagent is the limiting reagent, and completely reacts. The size difference between the conjugate and excess smaller reagent is greater than that between the conjugate and any unreacted larger reagent, and therefore, separation will be easier.

(2) 5 nm Nanogold® reagents are multi-functional

Unlike the smaller 1.4 nm monofunctional Nanogold® reagents, each 5 nm gold nanoparticle may contain as many as 20 or more reactive groups. If you are using a 5 nm Nanogold® reagent to label a molecule containing more than one reactive group, then not only can the 5 nm Nanogold® react with a second biomolecule, but the second reactive group on a labeled biomolecule may then react with a second 5 nm Nanogold® reagent. This process can produce extended oligomers, leading to the formation of intractable aggregates or precipitates.

Labeling Strategy

(1) Determine desired degree of labeling and reaction stoichiometry (ratio of 5 nm Nanogold® reagent : molecule to be labeled)

The best strategy for efficient Nanogold® labeling is to use an excess of the reagent that is more easily separated. The reagent that is more difficult to separate is the "limiting" reagent: it reacts completely, and therefore none is left unreacted that must be separated.

Which reagent is more easily separated depends upon the separation method. Most methods, including the simplest and best optimized, are based on size. Usually, it is easier to use an excess of the smaller of the two reagents, because the difference in size, and hence the resolution, will be greater between the conjugate and the smaller reagent than between the conjugate and the larger reagent.

To maximize your yield of monomeric conjugates and avoid forming excessively cross-linked oligomers or aggregates, use the following strategies:

(a) If possible, use a conjugation reaction that targets a unique functional group. For example, if the molecule you wish to label has several lysine residues (amines) but only one cysteine (thiol), use 5 nm Maleimido Nanogold® to label at the cysteine, rather than using 5 nm Amino Nanogold® to label at an amine.

(b) Take advantage of immobilization. If you can immobilize your target before labeling, you will prevent the 5 nm Nanogold® from cross-linking multiple conjugate biomolecules together. If you are using a 5 nm Nanogold® reagent to label a probe that binds a target on a non-reactive surface, allow the probe to bind to this surface, then apply the 5 nm Nanogold®. This approach also allows easy removal of excess 5 nm Nanogold® by washing.

(c) Use a reaction stoichiometry that favors your desired product and a compatible separation method: if you are labeling a larger molecule that has a single reactive group, use a small excess (2-fold to 3-fold) of 5 nm Nanogold®. Using excess 5 nm Nanogold® will favor reaction of biomolecules with unconjugated Nanogold® particles, reducing the fraction of reactions between a conjugate biomolecule and an already conjugated 5 nm Nanogold®, while making the conjugate biomolecule the limiting reagent and ensuring that it is completely labeled.

If you are labeling a smaller molecule with a single reactive group and require 1 : 1 conjugates (one Nanogold® per conjugate), also use a small excess (2-fold to 3-fold) of 5 nm Nanogold®, but use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography.

If you cannot use a unique functional group on the molecule you are labeling, adjust the reaction stochiometry to minimize excessive cross-linking and favor the formation of the desired product. Make sure that you can separate conjugates in a form and purity that meet the needs of your experiment: if either unconjugated 5 nm Nanogold® or unlabeled probe would interfere with your experiment, make sure that you can separate them once reaction is complete.

(i) If you are labeling a smaller molecule that has more than one reactive group and a conjugate with more than one conjugate molecule per 5 nm Nanogold® works for your experiment, use an excess of the smaller molecule to be labeled (five-fold to tenfold: use the smaller excess for molecules closer in size to the Nanogold®, and a larger excess for much smaller molecules).

(ii) If you are labeling a larger molecule with more than one reactive group and a conjugate with more than one 5 nm Nanogold® per conjugate works for your experiment, use an excess of 5 nm Nanogold® (five-fold to tenfold: use the smaller excess for molecules closer in size to the Nanogold®, and a larger excess for much larger molecules).

(iii) If you are labeling a smaller molecule with more than one reactive group and require 1 : 1 labeling (one 5 nm Nanogold® per conjugate), then use a small excess of the 5 nm Nanogold® (1.5 : 1 or 2 : 1). After it has reacted, the larger bound Nanogold® should hinder approach and reaction with a second Nanogold® sufficiently to favor the formation of enough monomeric 1 : 1 product to produce a usable yield. Use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography, that can separate unconjugated 5 nm Nanogold®. Any larger aggregates may be removed by centrifugation at low g to pellet them, or by filtration.

(iv) If you are labeling a larger molecule with more than one reactive group and require 1 : 1 labeling (one 5 nm Nanogold® per conjugate), then if possible, use a small excess of the molecule to be labeled (1.5 : 1 or 2 : 1). The larger conjugate biomolecule should hinder approach and reaction of the Nanogold® with a second conjugate biomolecule sufficiently to favor the formation of enough monomeric 1 : 1 product to produce a usable yield. Use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography. Any larger aggregates may be removed by centrifugation at low g to pellet them, or by filtration.

(2) Use a compatible a separation method

Nanogold® conjugates may be separated from unconjugated Nanogold® and unlabeled conjugate biomolecule using these methods:

(a) Centrifuge concentration (membrane filtration): If there is a large size difference between the molecule you plan to label and the 5 nm Nanogold® reagent, you can use a centrifuge filter or concentrator to remove the smaller of the reagents:

(i) If you are labeling a molecule with a molecular weight of 50,000 Da or less, a 100,000 molecular weight cut-off (MWCO) filter will retain the conjugate and any unconjugated 5 nm Nanogold® and filter out unlabeled biomolecule. For pure product, use an excess of the molecule to be labeled to ensure complete reaction of the 5 nm Nanogold®.

(ii) If you are labeling a molecule with a molecular weight of 1,000,000 Da or more, a 1,000,000 molecular weight cut-off (MWCO) filter will retain the conjugate and any unlabeled biomolecule and filter out unconjugated 5 nm Nanogold® reagent. For pure product, use an excess of the 5 nm Nanogold® reagent to ensure complete reaction of the molecule to be labeled.

(a) Gel filtration (size exclusion) liquid chromatography: If the size difference between the molecule you plan to label and the 5 nm Nanogold® reagent does not allow effective centrifuge filtration, or if you need to characterize the species formed by the reaction, gel filtration (size exclusion) liquid chromatography can effectively isolate conjugates while providing information on the sizes of the different reaction products.

If you plan to use gel filtration, use an excess of the smaller of the two reagents; this will ensure that the larger molecule, or limiting reagent, reacts completely, and chromatographic resolution between the conjugate and unreacted excess smaller reagent will be higher than that between conjugate and unreacted larger reagent. If the two reagents are similar in size, best results are usually obtained using a small excess (1.5 to 2-fold) of 5 nm Nanogold® reagent.

(i) If you are labeling a smaller molecule (usually with a molecular weight of 150,000 Da or less), use an excess (3-fold) of the molecule to be labeled; this will ensure complete reaction of the 5 nm Nanogold®, and unreacted smaller molecule will be more completely separated.

(ii) If you are labeling a larger molecule (usually with a molecular weight of 200,000 Da or more), use an excess (2-fold to 3-fold) of 5 nm Nanogold® reagent; the conjugate molecule will react completely, and unconjugated 5 nm Nanogold® reagent will be more completely separated.

In general, we find that agarose gels are more compatible with Nanogold® conjugates than acrylate gels. Use a gel where the expected size of the conjugate is near the upper end of the separation range, and the smaller of the two reagents is towards the lower end. Superose-6 (Cytiva), which has a MW fractionation range of 5×10^4 – 5×10^6 Da (exclusion limit 4×10^7 Da) effectively separates 5 nm Nanogold® conjugates, as both conjugates and unconjugated smaller molecules are eluted within the separation range and both may be resolved from any larger oligomers or aggregates. Other gels with appropriate separation characteristics include the larger molecular weight range Bio-Gel columns from Bio-Rad: Bio-Gel A-5m gel (MW separation range 10,000–5,000,000 Da), Bio-Gel A-15m gel (MW separation range 40,000–15,000,000 Da) and Bio-Gel A-50m gel (MW separation range 100,000–50,000,000 Da). Appropriate HPLC columns include TSKgel® SuperAW5000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

(c) Other chromatographic methods: other liquid chromatography modalities, such as ion exchange (IEC), reverse-phase or hydrophobic interaction chromatography (HIC), may be useful for labeling reactions where size and conjugate properties are incompatible with size-dependent separation. If you are using one of these methods, test a small portion of (i) unreacted 5 nm Nanogold® reagent and (ii) unlabeled biomolecule before reaction so that you know where each elutes and can identify the conjugate.

(d) Ammonium sulfate precipitation: this will precipitate proteins and conjugates, while leaving unconjugated 5 nm Nanogold® in solution is effective: it is effective when using excess 5 nm Nanogold® reagent. Since it is not size-dependent, it is useful for some reactions where the centrifuge filtration or gel filtration liquid chromatography do not provide efficient separation, for example preparation of a 1 : 1 conjugate with a smaller molecule using excess 5 nm Nanogold® reagent.

(e) Gel electrophoresis: may be useful for analytical separations. Note that gel shifts alone may differ from those expected on the basis of molecular weight and may not be reliable indicators of the success or failure of a conjugation reaction. For best results:

(i) Use a non-reducing gel; thiols can degrade Nanogold® or its conjugates.

(ii) Run two lanes in parallel under identical conditions.

(iii) Divide the two lanes. Stain one lane using a protein stain such as Coomassie blue. Develop the other using a silver or gold enhancement reagent (do not use a protein silver stain, as this is a different chemistry).

Conjugate bands will be visualized on both gels. Unlabeled proteins will be visualized only by protein stain, and unconjugated 5 nm Nanogold® only by silver or gold enhancement.

Labeling Glycoproteins with 5 nm Amino Nanogold®

This section contains a general protocol for labeling glycoproteins or aldehyde-functionalized molecules using 5 nm Amino Nanogold®. This protocol may be used as a starting point for optimization of your labeling procedure.

5 nm Amino Nanogold® is reactive towards aldehydes, and may be used to label carbohydrate moieties of glycoproteins[2] as shown in figure 1 (above).

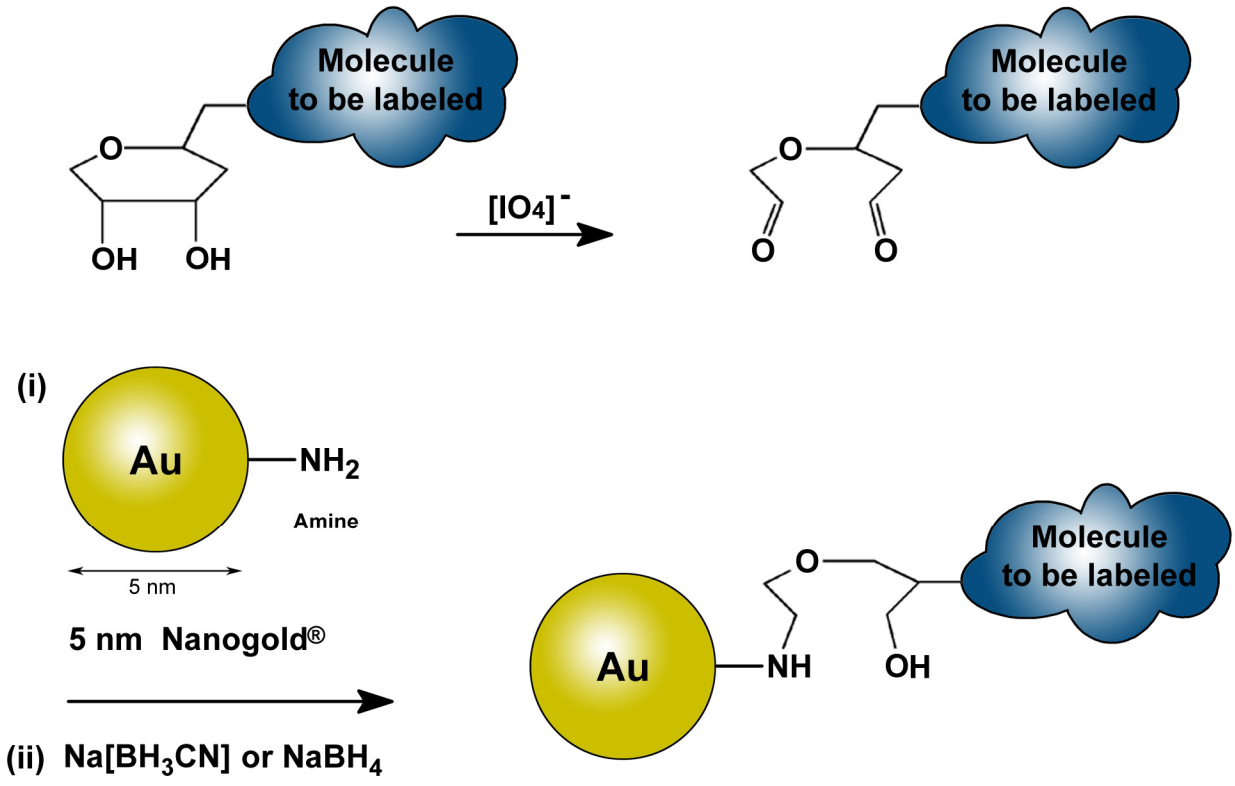

Figure 1: Labeling of a carbohydrate moiety with Monoamino Nanogold.[2]

Amount of protein to use

If you are labeling a protein or other biomolecule and plan to separate the Nanogold®-labeled product by centrifuge concentration (membrane filtration), gel filtration liquid chromatography or ammonium sulfate precipitation, prepare the following volumes of protein solution:

Proteins with a unique reactive amine – centrifuge concentration (membrane filtration) or gel filtration separation:

(a) If the protein to be labeled has just one reactive glucose residue or aldehyde and is larger than about 150,000 Da (150,000 MW) and you plan to use gel filtration separation, use an excess of 5 nm Amino Nanogold®. For proteins similar in size, use a small (2-fold) excess (i.e. 2.5 nmol of protein), and for much larger proteins (1,000,000 MW or larger) use a 5-fold excess (i.e. 1 nmol of protein).

(b) If the protein to be labeled has just one unique glucose residue or aldehyde and is smaller than about 150 kDa (150,000 MW), and you plan to use gel filtration separation, you should use an excess of the conjugate biomolecule, as this will ensure complete reaction of the 5 nm Amino Nanogold® (the limiting reagent) and easy removal of excess unreacted conjugate biomolecule. For biomolecules close in size to 5 nm Amino Nanogold®, use a small excess (2-fold, or 2.5 nmol of protein); for smaller biomolecules (less than 50,000 MW), use a larger excess (5-fold to 10-fold, or 0.5 – 1 nmol of protein).

However, if your conjugate biomolecule has only one reactive glucose residue or aldehyde and quantitative labeling or formation of 1 : 1 conjugates is your priority, use a small excess (1.5 to 2-fold) of 5 nm Amino Nanogold®. This will statistically favor the formation of 1 : 1 conjugates. However, if possible, you should use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity chromatography, to isolate the conjugate from excess unreacted 5 nm Amino Nanogold®.

Proteins with a unique reactive thiol – ammonium sulfate precipitation:

(c) Use an excess (3-fold to 5-fold) of 5 nm Amino Nanogold® to give a 1 : 1 ratio of 5 nm Nanogold® : protein. This should ensure the highest yield of Nanogold® conjugate while minimizing oligomer formation.

Proteins with more than one reactive thiol:

(d) If the protein to be labeled has multiple accessible glucose residues or aldehydes and is larger than about 200,000 Da, use an excess of 5 nm Amino Nanogold®. For proteins similar in size, use a small (2-fold) excess (i.e. 2.5 nmol of protein), and for much larger proteins (1,000,000 MW or larger) use a 5 to 10-fold excess (i.e. 0.5 to 1 nmol of protein). This will help avoid oligomerization and aggregation through multiple cross-linking.

(e) If the protein to be labeled has more than one accessible glucose residue or aldehyde and is smaller than about 150 kDa (150,000 MW): If minimizing aggregation is your priority, use an excess of the conjugate biomolecule, as this will reduce the probability of reaction at a second glucose residue or aldehyde. For biomolecules close in size to 5 nm Amino Nanogold®, use a small excess (2-fold); for smaller biomolecules (less than 50,000 MW), use a larger excess (5-fold to 10-fold).

If you are labeling an oligonucleotide and plan to separate the Nanogold®-labeled product by ethanol precipitation, use a 1 : 1 ratio of 5 nm Amino Nanogold® : oligonucleotide (i.e. 5 nmol). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

Procedure

To assist structural preservation, it is usually helpful to fix proteins before labeling. If glutaraldehyde or other aldehyde-containing fixing reagents are used, these should be quenched before labeling. This may be achieved by reacting with sodium borohydride (15-20 mole equivalents per glutaraldehyde) at 4°C for 1.5 h.[2] Alternatively, incubate the specimens for 5 minutes in 50 mM glycine solution in PBS (pH 7.4), or treat with ammonium chloride (50 mM).

The procedure below is a recent modification of our original procedure, which has been found to give more selective labeling.[5] Sugars must be oxidized to aldehydes for reaction with 5 nm Amino Nanogold® to take place. Sodium periodate at 1 mM, 0°C slectively cleaves only sialic acid. At 10 mM or higher concentrations, other sugars are cleaved. The concentration of the periodate used should be adjusted accordingly.

Oxidation:

1. Dissolve the glycoprotein in 0.01 - 0.1 M phosphate buffer, pH 7.0. Other buffers and pH values are acceptable, except that amine-containing bufers such as tris or glycine must be avoided.
2. Make a 10 mg/mL solution of sodium periodate in water, protect from light. Add to the protein to make a final concentration of 10 mM periodate (1 mM if labeling at sialic acid); protect from light.

3. React at room temperature for 15 - 30 minutes.
4. Sepatate the oxidized glycoprotein from excess periodate by gel filtration; optionally, the unreacted periodate may be quenched beforehand with 0.1 mL glycerol/mL of reaction mixture.

Alternatively, enzymes may be used to oxidize the diol groups (e.g. glucose or galactose oxidases). An example is given below - oxidase reaction for cell labeling of galactose.

Enzymatic oxidation:

1. Prepare 5 % cell suspension in the appropriate buffer. Avoid amine-containing buffers such as tris or glycine.
2. Add 0.05 units of vibrio cholerae neuraminidase (to remove sialic acid sugars to expose galactose as the terminal residue) and 5 units of galactose oxidase per mL of cell suspension.
3. Incubate for 60 minutes at 37°C.

Gold labeling:

1. Dissolve the antibody or protein to be labeled in 0.2 M sodium carbonate buffer, pH 9.6 or an alternative buffer, adjusted to pH 9.6 (1 mL).
2. Dissolve the 5 nm Amino Nanogold® in 1 mL of the same buffer, or an alternative buffer, adjusted to pH 9.6. If the reagent is slow to dissolve, vortex or sonicate the solution.

   If solubility is known to be an issue with the molecule you are labeling, solubility may be improved by predissolving the 5 nm Amino Nanogold® reagent in up to 10% of the final reaction volume (0.2 mL) of ispropanol or DMSO, then making up to 1 mL with buffer.
3. Add the reconstituted 5 nm Amino Nanogold® solution to the dissolved protein. Adjust the reaction volume to give a protein concentration of at least 1 nmol/mL (for a 150,000 MW protein, this is 0.15 mg/mL) to achieve optimum labeling. A membrane centrifugation unit may be used to reduce the volume of the reaction mixture; a 50,000 or 100,000 nominal molecular weight cut-off unit is recommended (e.g. Amicon Ultra-2, 100,000 MWCO, molecular weight cut-off, from Thermo Fisher or EMD Millipore) to ensure retention of the protein and Nanogold® reagent.
4. Incubate for 2 hours at room temperature. Reaction mixture may then be stored overnight (12-18 hours) at 4°C.
5. Add 10 microliters of 5 M sodium cyanoborohydride per mL of reaction (**CAUTION: Toxic - do this in a fume hood**). The 5 M solution is prepared in 1 N sodium hydroxide.
6. React for 30 minutes at room temperature.
7. Block unreacted aldehyde with 50 microliters of 1 M ethanolamine, pH 9.6 per mL of reaction mixture. A 1 M ethanolamine solution is prepared by the addition of 300 microliters of ethanolamine to 5 mL of water and adjusting the pH with concentrated hydrochlorid acid while on ice.
8. React for 30 minutes at room temperature.
9. Separate the unbound gold particles from the protein conjugate (see below).

The conventional procedure is given below:

1. If the labeling site is in the form of a carbohydrate group, it must be oxidized to produce aldehydes. Incubate specimen for 1.5 h at 4°C with 1000-2000 mole equivalents of sodium periodate per carbohydrate in 20 mM PIPES.Na.
2. Isolate oxidized glycoprotein by by dialysis or gel filtration chromatography to remove sodium periodate. Use a gel such as Amicon GH-25, which has an exclusion cut-off at molecular weight 3,000. As the eluent, use 20mM PIPES.Na, with 150 mM sodium chloride. The oxidizd glycoprotein will be eluted in the void volume as the first sharp peak in the trace. Combine the fractions containing protein; the total amount may be calculated from the optical density.
3. Dissolve the 5 nm Amino Nanogold® in 1 mL buffer. Solution is facilitated by first dissolving the reagent in 0.1 mL DMSO or isopropanol, then diluting to 1 mL with the buffer used for the reaction. Sufficient reagent is supplied to label up to 5 nmol of aldehydes; if you are using a smaller amount, use a proportionately smaller amount of 5 nm Amino Nanogold®. Once activated 5 nm Amino Nanogold® is reconstituted with water it should be used within one week.

4. Add the 5 nm Amino Nanogold® solution to the oxidized glycoprotein (6 to 10 mole equivalents percarbohydrate chain). Incubate for 1 hour at 4°C.
5. Reduce the Schiff base linkages with excess $NaBH_4$; use 500-1000 mole equivalents per carbohydrate chain and allow to react for 30 minutes at 4°C; then quench with excess acetone.
6. Separate the unbound gold particles from the labeled glycoprotein using gel exclusion chromatography. The NANOGOLD® conjugate may be effectively isolated using a medium such as Pharmacia Superose 6 or 12 (which fractionate a wide range of molecular weights) or Amicon GCL-90 (which excludes molecules of mass 30,000 or greater). Concentrate the reaction mixture to a suitably small volume using membrane centrifugation (e.g. Amicon Centricon-30 system). Elute with 0.25 M ammonium acetate (nominal pH 7.7); monitor at 280 nm. The first, faintly colored peak is the conjugate, while the second, darker band is unbound 5 nm Amino Nanogold®. For even higher purity, repeat this process one time.

Conjugate separation:

Centrifuge concentration (membrane filtration): use (a) when labeling molecules 50,000 Da or lower molecular weight; use a 100,000 molecular weight cut-off (MWCO) centrifuge concentrator, or (b) when labeling molecules 1,000,000 Da or higher molecular weight; use a 1,000,000 molecular weight cut-off (MWCO) centrifuge concentrator. Repeat twice for higher purity. Resuspend the concentrate in 2 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

Ammonium sulfate precipitation: After two hours reaction at room temperature, add saturated ammonium sulfate to 38% of total volume (a 2 mL reaction volume will require 1.226 mL of saturated ammonium sulfate solution). Agitate gently, then centrifuge for 10 minutes at 5000 X g. Remove the supernatant and resuspend the residue in 2 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

Gel filtration (size exclusion) liquid chromatography: use a gel with an appropriate size fractionation range that will efficiently separate your labeled protein from smaller molecules. Choose a separation matrix with a separation range such that the unconjugated protein elutes towards the lower end of the range. For example, small molecules (molecular weights less than 10,000) may be separated using gels such as Superdex-75 or Superose-12 (Cytiva). For larger proteins with molecular weights greater than 100,000, appropriate gels include Superose 6 (Cytiva), and Bio-Gel A-1.5m, A-5m, A-15m or A-50m (Bio-Rad). Appropriate HPLC columns include TSKgel® SuperAW5000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

Concentrate the reaction mixture to a suitably small volume for injection using membrane centrifugation (e.g. Amicon Ultra-2, 50,000 or 100,000 MWCO). Elute with 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS); the first, red peak or shoulder is the conjugate, while the second, usually colorless band is excess unlabeled proteins. For even higher purity, repeat this process one more time.

Oligonucleotide conjugates may be separated by ethanol precipitation.

Labeling Other Molecules with 5 nm Amino Nanogold®

5 nm Amino Nanogold® may be used to label other groups if it is used with a suitable cross-linker. As an example, the use of bis (sulfosuccinimidyl) suberate (BS3)as a cross linker to label a protein with a primary amine is described below.[6] The reaction works best in concentrated protein solutions (i.e. smaller reaction volumes).

1. Dissolve the protein in 0.02 M sodium phosphate with 150 mM sodium chloride at pH 7.4 (0.5 mL).
2. Dissolve the 5 nm Amino Nanogold® in buffer. Solution is facilitated by first dissolving the reagent in 0.1 mL DMSO or isopropanol, then diluting with 0.02 M phosphate buffer with 150 mM NaCl, pH 7.4. Sufficient reagent is supplied to label up to 1 nmol of sites; if you are using a smaller amount, use a proportionately smaller amount of 5 nm Amino Nanogold®. Once 5 nm Amino Nanogold® is reconstituted with water it should be used within one week.
3. Add the 5 nm Amino Nanogold® solution to the protein (6 to 10 mole equivalents per mole equivalent amine labeling site). Mix thoroughly.
4. Dissolve BS^3 in DMSO (0.1 mL) and dilute to 2 ml with 0.02 M phosphate buffer with 150 mM NACl, pH 7.4. Use sufficient BS^3 to give a concentration of 1-2 mM in the reaction mixture (0.5 mg). Once dissolved, the cross-linking reagent should be used immediately. Hydrolysis will occur within a few hours.
5. Add the BS solution to the protein / 5 nm Amino Nanogold® solution, and incubate for 10 mins at room temperature.

6. Quench with excess 10 mM tris buffer at pH 7.0.
7. Separate the unbound gold particles from the labeled glycoprotein using membrane centrifuge concentration, ammonium sulfate precipitation or gel filtration liquid chromatography as discussed previously.

Other cross-linking reagents may be used to link the 5 nm Amino Nanogold® to other functionalities. The buffer and reagent concentrations used for this reaction, and the incubation time, may be varied according to the nature of the molecule under study.

Characterization of 5 nm Nanogold® Conjugates

The purified Nanogold® conjugated peptide, protein or oligonucleotide is normally red colored at a high concentration, and can be detected by UV-Vis spectroscopy in the wavelength range 250 - 800 nm. Unlike the UV-Vis spectrum of unlabeled peptide, protein or oligonucleotide, which is usually near or at baseline from 300 - 800 nm, the absorption spectra of Nanogold® conjugates have significant absorption over the range of 300 - 800 nm, with an absorption maximum (λ_{max}) close to 520 nm (the plasmon resonance).

Estimation of Degree of Labeling (DOL): To calculate the amount of 5 nm Nanogold®, measure the absorption at the λ_{max} given in the Product Specifications, and at either 280 nm (for proteins) or 260 nm (for oligonucleotides). Use the absorbance at λ_{max} to calculate the molar concentration of 5 nm Nanogold®, using the molar extinction coefficient of 1.2×10^7 $M^{-1}cm^{-1}$. Next, calculate the contribution from the 5 nm Nanogold® to the absorbance at 280 nm (for proteins) or 260 nm (for oligonucleotides). To do this, multiply the observed absorbance at λ_{max} by the appropriate ratio of the Optical Densities given in the Product Specifications: $\gamma_{gold\ (280nm/\lambda max)}$ ($OD_{280nm}/OD\lambda_{max}$) for proteins, or $\gamma_{gold\ (260nm/\lambda max)}$ ($OD_{260nm}/OD\lambda_{max}$) for oligonucleotides. Subtract the absorption due to Nanogold® from the measured absorbance at 280 nm; the result is the absorbance of the protein or oligonucleotide. Divide this result by the extinction coefficient at 280 nm (ε_{280nm}) or 260 nm (ε_{260nm}) to calculate the molar concentration of the protein or oligonucleotide respectively.

$[\text{Nanogold}^{®}] = [A_{\lambda max}]/1.2 \times 10^7$

$[\text{Protein}] = [A_{280nm} - \gamma_{gold\ (280nm/\lambda max)} \times A_{\lambda max}]/\varepsilon_{protein\ at\ 280\ nm}$

or

$[\text{Nucleic Acid}] = [A_{280nm} - \gamma_{gold\ (280nm/\lambda max)} \times A_{\lambda max}]/\varepsilon_{oligonucleotide\ at\ 260\ nm}$

$DOL = [\text{Nanogold}^{®}]/[\text{Protein}]$

or

$DOL = [\text{Nanogold}^{®}]/[\text{oligonucleotide}]$

Characterization by Gel Electrophoresis: Purified Nanogold® conjugates or Nanogold® conjugate mixtures may also be characterized using SDS gel, native gel or agarose gel. For best results, follow the procedure below:

1. Use a gel with two panels or lanes. Load purified Nanogold® conjugate, or Nanogold® conjugate mixture with unlabeled peptide, protein or oligonucleotide and unreacted Nanogold® reagent into the left panel of the gel.
2. Duplicate the loading in the same sequence and amounts into the right panel.
3. Run the gel to reach separation. Nanogold® has a molecular weight of about 15,000 dalton and negligible charge, and contributes little to the charges of labeled peptides, proteins or polynucleotides. **Caution**: Nanogold® conjugates and Nanogold® reagents should not be heated with ß-mercaptoethanol before loading onto gels as ß-mercaptoethanol degrades Nanogold® particles during incubation.
4. After running the gel to reach separation, cut the gel in the middle to separate the two lanes.
5. Wash one panel with deionized water for 3 x 15 minutes, then incubate this panel with LI silver™ (Nanoprobes Catalog #2013-250 mL) for 10 minutes. Wash with deionized water for 4 x 5 minutes and continue overnight. The Nanogold® conjugate and Nanogold® reagent bands will become brown in color upon incubation with LI Silver.
6. The other panel should be stained either with Coomassie stain (for proteins) or nucleic acid stains (for nucleic acids). Nanogold® conjugates with these molecules and unlabeled peptide, protein or nucleic acid will be stained.

Nanogold® conjugate bands will be stained by both LI Silver™ and Coomassie or nucleic acid stains.

General Considerations for Using 5 nm Nanogold® Reagents

- 5 nm Nanogold® is an extremely uniform 5 nm diameter gold particle (±10%).
- 5 nm Nanogold® is covalently attached to the peptide, protein, polycucleotide or live cells after click reactions
- 5 nm Nanogold® conjugates contain no aggregates. This is in sharp contrast to other colloidal gold conjugates that usually are prepared by centrifugation to remove the largest aggregates and frequently contain smaller aggregates.
- 5 nm Nanogold® particles do not have affinity to proteins as do colloidal golds. This reduces background and false labeling.
- 5 nm Nanogold® develops better with silver than do most other colloidal golds giving it higher sensitivity. Both silver and gold enhancement can be used to enlarge Nanogold® to desirable sizes for electron and light microscopy, gel and blot detection.

Using Stains with 5 nm Nanogold®

5 nm Nanogold® particles are relatively small, and in thick sections or particularly dense tissue, over staining with OsO_4, uranyl acetate or lead citrate may tend to obscure direct visualization of individual Nanogold® particles. Three recommendations for improved visibility of Nanogold® are:

1. Use of reduced amounts or concentrations of usual stains.
2. Use of lower atomic number stains such as Nanoprobes NanoVan™, a Vanadium based stain.[6]
3. Enhancement of Nanogold® using silver or gold enhancers.

Considerations for Direct Viewing of 5 nm Nanogold® in the Electron Microscope

Although 5 nm Nanogold® is larger and more readily visualized than our smaller 1.4 nm Nanogold®, silver or gold enhancement may be helpful for a good signal in the electron microscope, especially in the presence of electron-dense counterstains (see below).

5 nm Nanogold® provides improved resolution and smaller probe size over colloidal gold antibody products. Several suggestions for optimum visualization follow:

1. Before you start a project with Nanogold® it is helpful to see it so you know what to look for. Dilute the Nanogold® stock 1:5 and apply 4 µL to a grid for 1 minute. Wick the drop and wash with deionized water 4 times.
2. View Nanogold® at 100,000 X magnification with 10 X binoculars for a final magnification of 1,000,000 X. Turn the emission up full and adjust the condenser for maximum illumination.
3. The alignment of the microscope should be in order to give 0.3 nm resolution. Although the scope should be well aligned, you may be able to skip this step if you do step 4.
4. Objective stigmators should be optimally set at 100,000 X. Even if the rest of the microscope optics are not perfectly aligned, adjustment of the objective stigmators may compensate and give the required resolution. You may want to follow your local protocol for this alignment but since it is important, a brief protocol is given here:
    a. At 100,000 X (1 X 10^6 with binoculars), over focus, under focus, then set the objective lens to in focus. This is where there is the least amount of detail seen.
    b. Adjust each objective stigmator to give the least amount of detail in the image.
    c. Repeat steps a and b until the in focus image contains virtually no contrast, no wormy details, and gives a flat featureless image.
5. Now underfocus slightly, move to a fresh area, and you should see small black dots of 5 nm size. This is the Nanogold®. For the 1:5 dilution suggested, there should be about 2 to 5 gold spots on the small viewing screen used with the binoculars. Contrast and visibility of the gold particles is best at 0.2 - 0.5 m defocus, and is much worse at typical defocus values of 1.5 - 2.0 m commonly used for protein molecular imaging.

6. In order to operate at high magnification with high beam current, thin carbon film over fenestrated holey film is recommended. Alternatively, thin carbon or 0.2% Formvar over a 1000 mesh grid is acceptable. Many plastic supports are unstable under these conditions of high magnification/high beam current and carbon is therefore preferred. Contrast is best using thinner films and thinner sections.
7. Once you have seen Nanogold® you may now be able to reduce the beam current and obtain better images on film. For direct viewing with the binoculars reduction in magnification from 1,000,000 X to 50,000 X or 30,000 X makes the Nanogold® more difficult to observe. It is recommended to view at 1,000,000 X, with maximum beam current, align the objective stigmators, and then move to a fresh area, reduce the beam, and record on film.
8. If the demands of high resolution are too taxing or your sample has an interfering stain, a very good result may be obtained using silver or gold enhancement to give particles easily seen at lower magnification.

Electron Microscopy Immunolabeling with 5 nm Nanogold® Conjugates

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.

Cells in Suspension

1. Optional fixing of cells: e.g., with glutaraldehyde (0.05 - 1% for 15 minutes) in PBS. Do not use Tris buffer since this contains an amine. After fixation, centrifuge cells (e.g. 1 mL at 10^7 cells/ml) at 300 X g, 5 minutes; discard supernatant; resuspend in 1 mL buffer. Repeat this washing (centrifugation and resuspension) 2 times.
2. Incubate cells with 0.02 M glycine in PBS (5 mins). Centrifuge, then resuspend cells in PBS-BSA buffer (specified below) for 5 minutes.
3. Wash cells using PBS-BSA as described in step 1 (2 X 5 mins). Resuspend in 1 mL Buffer 1.
4. Place 50 - 200 µL of cells into Eppendorf tube. Dilute 5 nm Nanogold® conjugate ~ 50 times in PBS-BSA buffer and add 30 µL to cells; incubate for 30 minutes with occasional shaking (do not create bubbles which will denature proteins).
5. Wash cells in PBS-BSA as described in step 1 (2 X 5 mins).
6. Fix cells and antibodies using a final concentration of 1% glutaraldehyde in PBS for 15 minutes. Then remove fixative by washing with buffer 1 (3 X 5 mins).

PBS-BSA Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
0.5% BSA
0.1% gelatin (high purity)
Optional, may reduce background:
0.5 M NaCl
0.05% Tween 20

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

Negative staining may be used for electron microscopy of small structures or single molecules which are not embedded. Negative stain must be applied after the silver enhancement. NanoVan™ negative stain is specially formulated for use with Nanogold® reagents; it is based on vanadium, which gives a lighter stain than uranium, lead or tungsten-based negative stains and allows easier visualization of 5 nm Nanogold® particles with little or no silver enhancement.[6]

CAUTION: 5 nm Nanogold® particles degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

Thin Sections

Labeling with 5 nm Nanogold® may be performed before or after embedding.[7,8] Labeling before embedding and sectioning (the pre-embedding method) is used for the study of surface antigens, particularly small organisms such as viruses budding from host cells. It

gives good preservation of cellular structure, and subsequent staining usually produces high contrast for study of the cellular details. Labeling after embedding and sectioning (the post-embedding method) allows the antibody access to the interior of the cells, and is used to label both exterior and interior features. The procedures for both methods are described below.

Thin sections mounted on grids are floated on drops of solutions on parafilm or in well plates. Hydrophobic resins usually require pre-etching.

CAUTION: Nanogold particles degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

PROCEDURE FOR PRE-EMBEDDING METHOD:[7]

1. Float on a drop of water for 5 - 10 minutes.
2. Incubate cells with 1% bovine serum albumin in PBS buffer at pH 7.4 for 5 minutes; this blocks any non-specific protein binding sites and minimizes non-specific antibody binding.
3. Rinse with PBS-BSA (1 min).
4. Incubate with 5 nm Nanogold® conjugate diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum from the same species as the 5 nm Nanogold® labeled antibody, for 10 minutes to 1 hour at room temperature.
5. Rinse with PBS-BSA (3 X 1 min), then PBS (3 X 1 min).
6. Postfix with 1% glutaraldehyde in PBS (10 mins).
7. Rinse in deionized water (2 X 5 min).
8. Dehydrate and embed according to usual procedure. Use of a low-temperature resin (e.g. Lowicryl) is recommended.
9. Stain (uranyl acetate, lead citrate or other positive staining reagent) as usual before examination.

Silver enhancement may be performed before or after embedding (see below); it should be completed before postfixing or staining with osmium tetroxide, uranyl acetate or similar reagents is carried out.

PROCEDURE FOR POST-EMBEDDING METHOD:[7]

1. Prepare sections on plastic or carbon-coated nickel grid. Float on a drop of water for 5 - 10 minutes.
2. Incubate with 1% solution of bovine serum albumin in PBS buffer at pH 7.4 for 5 minutes to block non-specific protein binding sites.
3. Rinse with PBS-BSA (1 min).
4. Incubate with 5 nm Nanogold® conjugate diluted 1/40 - 1/200 in PBS-BSA with 1% normal serum from the same species as the 5 nm Nanogold® labeled antibody, for 10 minutes to 1 hour at room temperature.
5. Rinse with PBS (3 X 1 min).
6. Postfix with 1% glutaraldehyde in PBS at room temperature (3 mins).
7. Rinse in deionized water for (2 X 5 min).
8. If desired, contrast sections with uranyl acetate and/or lead citrate before examination.

Silver enhancement may also be used to render the 5 nm Nanogold® particles more easily visible (see below), especially if stains such as uranyl acetate or lead citrate are applied. If used, it should be completed before these stains are applied.

PBS-BSA Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
0.5% BSA
0.1% gelatin (high purity)
Optional, may reduce background:

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

0.5 M NaCl
0.05% Tween 20

Silver or Gold Enhancement of 5 nm Nanogold® for EM

Silver enhancement

5 nm Nanogold® will nucleate silver deposition resulting in a dense particle 2-40 nm in size or larger, depending on development time. In pre-embedding labeling procedures, silver enhancement may be performed before or after embedding. Silver enhancement must be completed before any staining reagents such as osmium tetroxide, lead citrate or uranyl acetate are applied, since these will nucleate silver deposition in the same manner as gold and produce non-specific staining. With 5 nm Nanogold® reagents, low-temperature resins (e.g. Lowicryl) should be used and the specimens kept at or below room temperature until after silver development has been completed. Silver enhancement is recommended for applications of 5 nm Nanogold® in which these stains are to be used, otherwise the 5 nm Nanogold® particles may be difficult to visualize against the stain.

Best results in the EM may be obtained using HQ Silver™, which is formulated to give slower, more controllable particle growth and uniform particle size distribution.[9] Our LI Silver™ kit is convenient and not light sensitive, and suitable for all applications.

Specimens must be thoroughly rinsed with deionized water before silver enhancement reagents are applied. This is because the buffers used for antibody incubations and washes contain chloride ions and other anions which form insoluble precipitates with silver. These are often light-sensitive and will give non-specific staining. To prepare the developer, mix equal amounts of the enhancer and initiator immediately before use. 5 nm Nanogold® will nucleate silver deposition resulting in a dense particle 2-20 nm in size or larger depending on development time. Use nickel grids (not copper).

The procedure for immunolabeling should be followed up to step 6 as described above. Silver enhancement is then performed as follows:

1. Rinse with deionized water (2 X 5 mins).
2. OPTIONAL (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 X 5 mins).
3. Float grid with specimen on freshly mixed developer for 1-8 minutes, 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 6 mins gives 15-40 nm round particles.
4. Rinse with deionized water (3 X 1 min).
5. Mount and stain as usual.

Fixing with osmium tetroxide may cause some loss of silver; if this is found to be a problem, slightly longer development times may be appropriate.

NOTE: Treatment with osmium tetroxide followed by uranyl acetate staining can lead to much more drastic loss of the silver-enhanced 5 nm Nanogold® particles. This may be prevented by gold toning:[10]

1. After silver enhancement, wash thoroughly with deionized water.
2. 0.05% gold chloride: 10 minutes at 4°C.
3. Wash with deionized water.
4. 0.5% oxalic acid: 2 mins at room temperature.
5. 1% sodium thiosulfate (freshly made) for 1 hour.
6. Wash thoroughly with deionized water and embed according to usual procedure.

Gold Enhancement

The following procedure has been found to give excellent results for pre-embedding immunolabeling using GoldEnhance™ EM Plus, and also works well with GoldEnhance™ EM.[11] Follow the procedure for immunolabeling up to step 4 (Nanogold® conjugate incubation) as described above, then conduct gold enhancement as follows:

1. Wash three times in PBST.
2. Fix 1 hour in 1.2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 5 % sucrose.

3. Wash two times with water.
4. Enhance using GoldEnhance EM Plus (6 minutes).
5. Post-fix in 2 % osmium tetroxide and 2 % potassium ferricyanide in the same buffer.
6. Dehydrate, embed in Epon 812, section and stain with lead citrate before examination in the electron microscope.

Immunolabeling and Silver or Gold Enhancement with 5 nm Nanogold® for Light Microscopy

Light Microscopy with Silver Enhancement

Nanoprobes LI Silver™ kit is simple, convenient and not light sensitive, and suitable for all applications Features labeled with 5 nm Nanogold® will be stained black in the light microscope upon silver enhancement. Different development times should be tried to determine which is best for your experiment. The immunolabeling procedure is similar to that for EM; an example is given below.

Samples must be rinsed with deionized water before silver enhancement. This is because the reagent contains silver ions in solution, which react to form a precipitate with chloride, phosphate and other anions which are common components of buffer solutions.

1. Spin cells onto slides using Cytospin, or use paraffin section.
2. Incubate with 1% w/v bovine serum albumin or in PBS (PBS-BSA) or 1% nonfat dried milk in PBS (PBS-Milk) for 10 minutes to block non-specific protein binding sites.
3. Rinse with PBS-BSA or PBS-Milk (3 X 2 min).
4. Incubate with 5 nm Nanogold® reagent diluted 1/40 - 1/200 in PBS-BSA with 1% normal serum from the same species as the 5 nm Nanogold® reagent, for 1 hour at room temperature.
5. Rinse with PBS (3 X 5 min).
6. Postfix with 1% glutaraldehyde in PBS at room temperature (3 mins).
7. Rinse with deionized water (3 X 1 min).
8. OPTIONAL (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 X 5 mins).
9. Develop specimen with freshly mixed developer for 5-20 minutes, or as directed in the instructions for the silver reagent. 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 7.4
0.5% BSA
0.1% gelatin (high purity)

Optional, may reduce background:

0.5 M NaCl
0.05% Tween 20

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

To obtain an especially dark silver signal, the silver enhancement may be repeated with a freshly mixed portion of developer.

Light Microscopy with Gold Enhancement

Nanoprobes GoldEnhance™ LM (catalog number 2112-28ML) is optimized for light microscopy. Features labeled with 5 nm Nanogold® will be stained black or purple in the light microscope upon silver enhancement. Optimum development times vary among different types of specimens, so different development times should be tried to determine which is best.

Immunolabeling should be completed up to step 6 as described above, then conduct gold enhancement as follows:

1. Wash two times with water.
2. Enhance using GoldEnhance™ LM (4 to 12 minutes).
3. Rinse with deionized water (2 X 5 mins).
4. The specimen may now be stained if desired before examination, with usual reagents.

IMMUNOBLOTTING

Immunoblotting with Gold Enhancement (recommended)

For more details and illustrations, see our newsletter article on immunoblotting with Nanogold® conjugates and GoldEnhance™ on our web site (https://www.nanoprobes.com/newsletters/Vol8_Iss10.html#2).

Suggested procedure:

REAGENTS AND EQUIPMENT:

- Phosphate buffered saline (PBS): 20 mM sodium phosphate buffer pH 7.4 and 150 mM NaCl.
- Specific antigen (target protein or other biomolecule).
- Nitrocellulose (NC) membrane 0.2 µm pore size.
- Blotting Paper to wick membrane dry.
- Orbital Shaker
- Washing buffer (TBS-Tween 20): 20 mM Tris pH 7.6, with 150 mM NaCl and 0.1 % Tween-20.
- Nonfat dried milk (Carnation)
- GoldEnhance™ EM (Nanoprobes Product No. 2113-8ML) or GoldEnhance™ Blots (Nanoprobes Product No. 2115-48ML).
- Specific Nanogold® antibody conjugate.

PROCEDURE:

Antigen Application:

1. Prepare antigen solutions with a series of dilutions (0.01mg/mL, 0.001mg/mL, 0.0005mg/mL, 0.0001 mg/mL, 0.00005 mg/mL, 0.00001 mg/mL and 0.000005 mg/mL) using PBS, pH7.4.
2. Pipette 1 µL of above solutions to a dry nitrocellulose membrane; prepare 2 duplicates as a negative control.
3. Negative control 1: No antigen, No antibody.
4. Negative control 2: No antigen with NG-conjugate incubation.
5. Air-dry for 30 minutes

Blocking:

6. Immerse membranes in 8 mL of TBS-Tween 20 for 5 minutes.
7. Block membranes in 8 mL of TBS-Tween 20 containing 5 % nonfat dried milk for 30 minutes at room temperature.

Binding of Nanogold® antibody conjugate:

8. Dilute Nanogold® antibody conjugate in TBS-Tween20 containing 1% nonfat dried milk to 4 µg/mL (1:20 Dilution: 300 µL conjugate + 5.30 ml TBS-gelatin containing 1% nonfat dried milk).
9. Incubate the membranes in 8 mL of diluted conjugate solution for 30 minutes at room temperature.
10. Incubate the control membrane in 8 mL of TBS-Tween20 containing 1% nonfat dried milk for 30 minutes at room temperature.

Autometallographic Detection:

11. Wash membranes three times for 3 min each in 8 mL of TBS-Tween 20. Wash membranes thoroughly in 8 mL of deionized water (4 x 3 minutes). Make sure strips are washed separately according to what they are incubated in (strips incubated in one lot of a conjugate are washed in a separate dish from strips that are incubated in TBS-Tween 20 with 1% nonfat dried milk without conjugate, strips incubated in different lots are washed separately).
12. Perform gold enhancement according to instructions (mix solutions A and B, wait 5 minutes, then add C and D).
13. Record the number of observed spots and time when the spots appear. Record the time when background appears on the control membrane.

14. After 15 minutes, remove the enhancement solution. Rinse membranes with water (3 x 3 minutes) and air-dry for storage.

Tween-20 and nonfat dried milk should also improve the performance of Nanogold® conjugates used with silver enhancement.

Immunoblotting with silver enhancement

The basic procedure for gold immunoblotting has been described by Moeremans et al,[12] which may be followed. For best results, the membrane should be hydrated before use by simmering in gently boiling water for 15 minutes. Best results are obtained when the antigen is applied using a 1 µL capillary tube. The procedure for immunoblots is as follows, if the 5 nm Nanogold® conjugate is the primary antibody:

1. Spot 1 µL dilutions of the antigen in buffer 4 onto hydrated nitrocellulose membrane. Use an antigen concentration range from 100 to 0.01 pg / µL.
2. Block with buffer 1 for 30 minutes at 37°C.
3. Rinse with buffer 1 (3 X 10 mins).
4. Incubate with a 1/100 to 1/200 dilution of the 5 nm Nanogold® reagent in buffer 2 for 2 hours at room temperature.
5. Rinse with buffer 3 (3 X 5 mins), then buffer 4 (2 X 5 mins).
6. OPTIONAL (may improve sensitivity): Postfix with glutaraldehyde, 1% in buffer 4 (10 mins).
7. Rinse with deionized water (2 X 5 mins).
8. OPTIONAL (may reduce background): Rinse with 0.05 M EDTA at pH 4.5 (5 mins).
9. Develop with freshly mixed silver developer for 20-25 minutes or as directed in the instructions for the silver reagent, twice. Rinse thoroughly with deionized water between developments to remove all the reagent.
10. Rinse several times with deionized water.

CAUTION: 5 nm Nanogold® particles degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

Buffer 1:
20 mM phosphate
150 mM NaCl
pH 7.4
4% BSA (bovine serum albumin)
2 mM sodium azide (NaN_3)

Buffer 2:
20 mM phosphate
150 mM NaCl
pH 7.4
0.8% BSA
1% normal serum; use serum of the host animal for the 5 nm Nanogold® antibody
0.1% gelatin (Type B, approx. 60 bloom)
Optional, may reduce background:
0.5 M NaCl
0.05% Tween 20

Buffer 3:
20 mM phosphate
150 mM NaCl
pH 7.4
0.8% BSA (bovine serum albumin)
2 mM sodium azide (NaN_3)

Buffer 4 (PBS):
20 mM phosphate
150 mM NaCl
pH 7.4

Other procedures may be used; for example the 5 nm Nanogold® reagent may be used as a tertiary labeled antibody, or a custom 5 nm Nanogold® conjugate may be the primary antibody. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.

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