# Fluorescein - FLUORONANOGOLD™* - Streptavidin

Nanoprobes

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## PRODUCT INFORMATION

### FLUORONANOGOLD™-FLUORESCEIN-GOLD-STREPTAVIDIN CONJUGATE

| | |
|---|---|
| Product Name: | FLUORONANOGOLD-Streptavidin conjugate |
| Catalog Number: | 7016 |
| Appearance: | Fluorescent pale greenish-yellow solution |
| Revision: | 1.6 (April 2003) |

Congratulations on your acquisition of a revolutionary new immunocytochemical reagent: FLUORONANOGOLD-Streptavidin. This unique histochemical probe is tagged with a molecular label which contains both the 1.4 nm NANOGOLD® particle and fluorescein, enabling both fluorescence and electron microscope observation of a sample stained in a single labeling procedure. This probe is smaller than a whole IgG molecule, does not aggregate, and fluorescence quenching due to the gold particle is negligible.

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**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.

*Patented technology.

**PRODUCT INFORMATION**

FLUORONANOGOLD™ is a unique, newly developed immunoprobe. FLUORONANOGOLD™-Streptavidin consists of streptavidin conjugated to a novel molecular label which contains both fluorescein and the 1.4 nm NANOGOLD® particle.[1] Streptavidin labeled

with FLUORONANOGOLD™ is shown in Figure **1** (overleaf). In the fluorescence microscope, this probes may be used just like conventional fluorescein-labeled immunoprobes,[2] while in the electron microscope it is visualized in exactly the same manner as for NANOGOLD® reagents.[3] The covalent label linkage is stable indefinitely, and the attachment at a hinge thiol site ensures maximum preservation of native immunoreactivity. This reagent is supplied at a concentration of 0.08 mg/mL of Streptavidin dissolved in 20 mM phosphate buffered saline (150 mM NaCl) at ph 7.4, with 0.1 % BSA and 0.05 % sodium azide as preservatives. FLUORONANOGOLD™ conjugates should be stored at 2-8°C. DO NOT FREEZE.

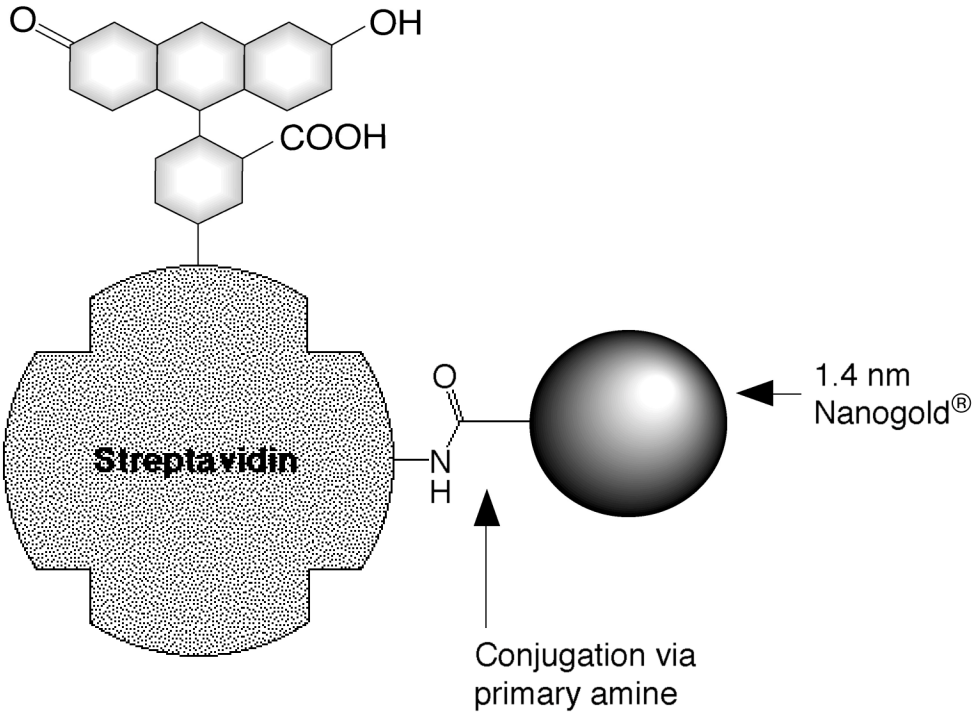


**Figure 1**: Streptavidin conjugated to Fluorescein and NANOGOLD® via primary amines to give FLUORONANOGOLD™.

## GENERAL CONSIDERATIONS FOR IMMUNOSTAINING WITH FLUORONANOGOLD™ REAGENTS

Basically, normal methodologies for each component of the label may be used successfully with FLUORONANOGOLD™ labeling agents. Due to some quenching of fluorescence by the gold particle, slightly higher concentrations of streptavidin are recommended for incubations. A blocking agent of 5% non-fat dried milk has been found to reduce background in some cases: this should be used before incubation with probe (in standard wash/blocking steps), and additionally, the FLUORONANOGOLD™- Streptavidin probe should be diluted in a solution also containing 5% non-fat dried milk before it is applied.

## PROPERTIES

FLUORONANOGOLD™ contains an extremely uniform 1.4 nm diameter gold particle (±10%).

Streptavidin - FLUORONANOGOLD™ is smaller than a single whole IgG molecule. It is not significantly larger than Fab'-NANOGOLD®, the smallest gold immunoprobe commercially available, and will penetrate and reach antigens inaccessible to other gold probes.

Streptavidin - FLUORONANOGOLD™ is chromatographically purified through gel filtration columns. There are absolutely no aggregates or other molecular weight impurities. This is in sharp contrast to colloidal gold conjugates which usually are prepared by centrifugation to remove the largest aggregates, and frequently contain smaller aggregates.

Close to 1 FLUORONANOGOLD™ label to 1 Streptavidin make this product distinct from the 0.2 - 10 variable stoichiometry of other colloidal gold antibody preparations.

FLUORONANOGOLD™ particles do not have affinity to proteins as do colloidal golds. This reduces background and false labeling.

FLUORONANOGOLD™ develops better with silver than do most colloidal golds, giving it higher sensitivity. Silver enhancement can be used to make the immunolabeling useful for electron microscopy, light microscopy, and immunoblotting with improved results.

## USING EM STAINS WITH Fluorescein - FLUORONANOGOLD™

Because the 1.4 nm FLUORONANOGOLD™ particles are so small, over staining with $OsO_4$, uranyl acetate or lead citrate may tend to obscure direct visualization of individual NANOGOLD® particles. Four recommendations for improved visibility of FLUORONANOGOLD™ are:

1. Use of reduced amounts or concentrations of usual stains.
2. Use of lower atomic number stains such as NANOVAN™, a Vanadium based stain.[4]
3. Enhancement of FLUORONANOGOLD™ with silver developers, such as LI SILVER or HQ SILVER.
4. Enhancement of FLUORONANOGOLD™ with the gold developer, GOLDENHANCE™.

## THIOL CAUTION

NANOGOLD® particles experience loss of gold clusters (Nanogold) upon exposure to thiols such as ß-mercaptoethanol (BME) or dithiothreitol (DTT). Avoid use of thiol agents. If a reducing environment is needed, reduce the protein, then purify from the thiol agent by column chromatography. Use non-metallic columns, and include 5 mM EDTA with the eluent, since trace metals catalyze thiol oxidation back to disulfides; most thiols do not reoxidize within several hours to several days following this procedure. Then use the Alexa Fluor®-FLUORONANOGOLD™. If a reducing agent is absolutely required, use a non-thiol agent, such as TCEP (triscarboxyethyl phosphine).

## TEMPERATURE CAUTION

Although NANOGOLD® is stable under most conditions,[5] labeled specimens or conjugates may not be stable above 80°C for long periods. Best results are obtained at room temperature or 4°C. It is best to use silver or gold enhancement before procedures requiring temperatures above 37°C, such as baking, or use low temperature embedding media (e.g., Lowicryl) if labeling before embedding.[6]

## METHODS

Several publications describe the successful application of FLUORONANOGOLD™ for light and electron microscopy. These provide additional protocols, details and applications that may be helpful in obtaining the best results (Refs. 1, 14-18).

## IN SITU HYBRIDIZATION WITH FLUORONANOGOLD®-STREPTAVIDIN

Procedure (adapted from that of Hacker, G.W., et al.):[7,8]

*Practical considerations*: This is a robust and reliable technique for routine use. It is intended for biotinylated hybridization probes. Other types of reporter molecules may be demonstrated by application of a biotinylated linking antibody system. The sensitivity of Nanogold-silver ISH depends, to a large degree, at the dilution of FluoroNanogold-streptavidin and the duration of silver development applied. Careful adjustment of the protease predigestion is necessary. In some preparations, some degree of unwanted background staining in connective tissue is obtained. This is in part due to fixation and possible excessive protease treatment and can often be reduced by application of higher dilutions of FluoroNanogold-streptavidin.

Fluorescence microscopy should be performed before silver enhancement since the silver deposition process results in removal of fluorescence.

1. Deparaffinize sections from formaldehyde-fixed tissue in fresh xylene (2 times 15 min each).
2. Rinse and rehydrate in graded alcohols and distilled water (2-3 min each).
3. Soak in phosphate-buffered saline (PBS, 20mM, pH 7.6) for 3 min.
4. Incubate with 0.1 mg/mL proteinase K (code no. 1 373 196, from Boehringer Mannheim, Mannheim, FRG) in PBS at 37°C for about 8 min. The duration is critical and has to be tested very carefully, depending on tissue, fixation and other factors.
5. Rinse in 2 changes of PBS, 3 min.
6. Permeabilize with 0.3% Triton X-100 in PBS for 15 min.
7. Wash in PBS for 2 min.
8. Rinse in 2 changes of distilled water, dehydrate with graded alcohols (50%, 70%, 98% isopropanol) for 1 min each and air-dry the sections.
9. Prehybridize with 1:1 mixture of deionized formamide and 20% dextran sulfate in 2X SSC at 50 °C for 5 min.
10. Carefully shake off excess prehybridization block.
11. Add one drop of biotinylated DNA probe on the section and cover with a small coverslip. Avoid air bubbles.
12. Heat sections on heating block at 92-94 °C for 8-10 min to denature DNA.
13. Incubate in a moist chamber at 37°C overnight (or for at least 2 hours).
14. Post-hybridization washes (5 min each): 2 changes of 4X SSC (1st wash to remove coverslips), 2X SSC, 0.1X SSC, 0.05X SSC, and then distilled water.
15. Put slides into Lugol's iodine solution (Merck, Darmstadt, Germany) for 5 min.
16. Wash in tap water and then distilled water.
17. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless. Then wash in tap water for 5 min and distilled water for 2 min.
18. Immerse in PBS containing 0.1% fish gelatin (45% concentrate – Cat. No. G-7765, Sigma-Aldrich, Steinheim, Germany) and 0.1% Tween-20 for 5 min.
19. Incubate sections with FluoroNanogold-streptavidin diluted 1:200 to 1:500 in PBS containing 1% BSA at room temperature for 60 min.
20. Wash in 3 changes of PBS containing 0.1% fish gelatin and 0.1% Tween-20 for 5 min each.
21. Repeatedly wash in distilled water for at least 10 min altogether, the last 2 rinses in ultrapure water (EM-grade).

    The specimen may now be observed by fluorescence microscopy.

22. Perform silver acetate autometallography or GoldEnhance development (Nanoprobes, see Protocol 4).
23. Rinse carefully in tap water for at least 3 min. After silver amplification, sections can be counterstained with Nuclear Fast Red, dehydrated and mounted in Permount or in DPX (BDH Chemicals, Poole, UK). Do not use Eukitt.

Solutions:

Phosphate-buffered saline (PBS): 10X PBS (Mg2+ and Ca2+-free) pH 7.6: 11.36 g $Na_2HPO_4$, 2.72 g $KH_2PO_4$, 87.0 g NaCl in 800 mL distilled water. Adjust pH with concentrated NaOH and add distilled water to a final volume of 1 L.

Standard Sodium Citrate Buffer (SSC): 175.32 g NaCl and 88.23 g sodium citrate in 800 ml distilled water. Adjust pH with NaOH to 7.0 and add distilled water to a final volume of 1 L.

Silver Development:

1. Silver amplification: Place the slides vertically in a glass container (preferably with about 80 mL volume and up to 19 slides; Schiefferdecker-type) and cover them with the mixture of solutions A and B. Staining intensity can be checked in the light microscope during the amplification process, which usually takes about 5-20 min, depending on primary antibody or nucleic acid probe concentration, incubation conditions, and the amount of accessible antigen or nucleic acid sequence in question.
2. Stop enhancement by washing in distilled water (several changes).
3. After stopping the enhancement process, slides can be examined in a light microscope more carefully. If staining intensity is still too low, wash slides for one more time in double-distilled water and develop further in enhancement solution.

Reagents (Solutions A and B should be freshly prepared for every run):

Solution A: Dissolve 80 mg silver acetate (code 85140; Fluka, Buchs, Switzerland) in 40 mL of glass double-distilled water. (Silver acetate crystals can be dissolved by continuous stirring within about 15 min.)

Citrate buffer: Dissolve 23.5 g of trisodium citrate dihydrate and 25.5 g citric acid monohydrate in 850 mL or deionized or distilled water. This buffer can be kept at 4°C for at least 2-3 weeks. Before use, adjust to pH 3.8 with citric acid solution.

Solution B: Dissolve 200 mg hydroquinone in 40 mL citrate buffer.

Enhancement solution: Just before use, mix solution A with solution B.

Alternative autometallography procedure (using LI Silver, Nanoprobes):

1. Allow LI Silver to come to room temperature.
2. Mix equal amounts of A and B solutions of LI Silver (red and blue capped bottles).
3. Immediately cover sections with developer.
4. Keep in subdued light, but check sections occasionally, using eye or light microscope. Development usually takes 20 min to 45 min.
5. Stop development by rinsing in deionized water. Alternatively, a fixer may be used to stop development immediately. Use a photographic fixer (e.g., Agefix, Agfa Gevaert, FRG, diluted 1:20) (can be reused). Treat for ~1 min. Another alternative is a 2.5% aqueous solution of sodium thiosulfate.
6. If sections are overdeveloped, they may be "back-developed" by 0.2 % Farmer's solution (9 parts sodium thiosulfate + 1 part potassium ferricyanide; form by mixing 0.18 g $Na_2S_2O_3$ . 5 $H_2O$ + 0.02 g $K_3Fe(CN)_6$ in 100 ml water). Reversal may be halted with water.
7. Rinse slides carefully in tap water for at least 3 min. After silver amplification, sections can be counterstained with hematoxylin and eosin or nuclear fast red, dehydrated, and mounted in DPX (BDH Chemicals, UK).

## FLUORESCENCE MICROSCOPY IMMUNOLABELING WITH FLUORONANOGOLD™

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

The procedure below[2] describes an example of the use of a FLUORONANOGOLD™ conjugate as a secondary antibody probe. Dilutions of FluoroNanogold will vary with different procedures, but a 5-fold or 10-fold dilution is advisable as a starting point for most applications; for simultaneous electron microscopy labeling, a compromise between the optimum concentrations for fluorescence and electron microscopy maybe necessary. Other protocols and techniques used with fluorescently-labeled antibodies may also be used with FLUORONANOGOLD™. 10 mM biotin should be added to the third wash in step 8; this is because vacant biotin binding sites can quench the fluorescence of the FLUORONANOGOLD™ label, and should therefore be blocked before observation. It should also be noted that the fluorescence intensity of fluorescein is pH-dependent: it is maximized at pH 9.0 or higher, reducing to approximately 85 % at pH 7 and decreasing rapidly at lower pH values. Therefore, we recommend that the buffer used for the final wash should have a pH value of 7.4 or higher.

1. Fix cells in freshly-prepared 2 % formaldehyde in PBS for 15 mins at 20°C; alternatively, fix in 100 % methanol at -20°C for 3 minutes; if methanol fixation is used, skip to step 4.
2. Wash in PBS (3 x 10 mins).
3. Permeabilize in 0.2 % Triton X-100 plus 1 % normal serum (NS) in PBS at pH 7.3 for 5 minutes on ice.
4. Wash in PBS with 1 % NS (3 X 10 mins).
5. Incubate in the appropriate concentration of biotinylated primary antibody for 1 hour at room temperature in a humidified chamber. If using 22 mm X 22 mm square cover slips, 30 µL of diluted antibody is placed on the coverslip and the coverslip is inverted onto a glass slide. The slide is then placed in a humidified chamber which is incubated at room temperature. Alternatively, a tertiary labeling procedure may be used where the primary antibody is not biotinylated, but the second antibody is. If additional antibody incubation steps are used, rinse with PBS with 1 % NS (3 X 10 mins) after incubation.
6. Wash in PBS with 1 % NS + 5% non-fat dried milk (3 X 10 mins).

7. Incubate with FluoroNanogold™-Streptavidin reagent at a dilution of 1 : 5 to 1 : 10 (diluted in buffer containing 5% non-fat dried milk) for 1 hour in a humidified chamber at room temperature.
8. Wash in PBS (4 X 10 mins).
9. Mount coverslip with a drop of mounting medium. Observe as usual.

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

**ELECTRON MICROSCOPY WITH FLUORONANOGOLD™ - STREPTAVIDIN**

The procedures given in this section are complete immunolabeling procedures, and are also recommended for NANOGOLD™ conjugates.[3] If the specimen has already been labeled and observed by fluorescence microscopy, it requires only mounting, silver enhancement (if necessary) and negative staining according to your usual electron microscopy protocol before observation.

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**

If the cells are already labeled, mount, stain and observe as usual. If a different specimen is to be used, the procedure below is recommended:

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 which reacts with glutaraldehyde.
2. 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.
3. Incubate cells with 0.02 M glycine in PBS (5 mins). Centrifuge, then resuspend cells in PBS-milk buffer (specified below) or PBS containing 1 % BSA for 5 minutes.
4. Place 50 - 200 µl of cells into Eppendorf tube and add 5 - 10 µl of biotinylated primary antibody. Incubate 30 minutes with occasional shaking (do not create bubbles which will denature proteins). Alternatively, a tertiary labeling procedure may be used where the primary antibody is not biotinylated, but the second antibody is. If additional antibody incubation steps are used, rinse with PBS-milk buffer (3 X 10 mins) after incubation.
5. Wash cells using PBS-Milk as described in step 2 (2 X 5 mins). Resuspend in 1 ml PBS-Milk buffer.
6. Dilute FLUORONANOGOLD™-Streptavidin ~ 5 to 10 times in PBS-Milk buffer and add 30 µl to cells; incubate for 30 minutes with occasional shaking.
7. Wash cells in PBS buffer as described in step 2 (2 X 5 mins).
8. Fix cells and antibodies using a final concentration of 1% glutaraldehyde in PBS for 15 minutes. Then remove fixative by washing with PBS buffer (3 X 5 mins).

PBS-Milk Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
5% Non-fat dried milk (final concentration)

*Optional, may reduce background:*

0.5 M NaCl
0.05% Tween 20
0.1% gelatin (high purity)

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

## NEGATIVE STAINING

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;[4] it is based on vanadium, which gives a lighter stain than uranium, lead or tungsten-based negative stains and allows easier visualization of FLUORONANOGOLD™ particles with little or no silver enhancement.

Other procedures may be used; for example the NANOGOLD® reagent may be used as a tertiary labeled antibody in a system where a biotinylated secondary antibody is used with an unlabeled primary antibody. If additional antibody incubation steps are used, rinse with PBS-Milk (3 X 5 mins) after incubation.

### Thin Sections

Labeling with FluoroNanogold™-Streptavidin may be performed before (the pre-embedding method)[9,10] or after embedding and sectioning (the post-embedding method).[9,10] 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.

PROCEDURE FOR PRE-EMBEDDING METHOD:[9]

If specimen has already been labeled with FLUORONANOGOLD™, skip to step 9. If a fresh specimen is required for EM, the following procedure is recommended.

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 non-specific protein binding sites and minimizes non-specific antibody binding.
3. Incubate with biotinylated primary antibody, diluted at usual working concentration in PBS-milk buffer or PBS containing 1 % BSA (1 hour or usual time. Buffer formulations are given below). Alternatively, a tertiary labeling procedure may be used where the primary antibody is not biotinylated, but the second antibody is. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.
4. Rinse with PBS-Milk (3 X 1 min).
5. Incubate with FLUORONANOGOLD™-Streptavidin reagent diluted 1/5 - 1/20 in PBS- Milk with 1 % normal serum for 10 minutes to 1 hour at room temperature.
6. Rinse with PBS - Milk (3 X 1 min), then PBS (3 X 1 min).
7. Postfix with 1 % glutaraldehyde in PBS (10 mins).
8. Rinse in deionized water (2 X 5 min).
9. Perform silver or gold enhancement (e.g., HQ Silver™ or GoldEnhance™), as specified in those product directions.
10. Dehydrate and embed according to usual procedure.
11. Stain (uranyl acetate, lead citrate or other staining reagent) as usual before examination.

PROCEDURE FOR POST-EMBEDDING METHOD:[9]

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. Incubate with biotinylated primary antibody, diluted at usual working concentration in PBS-Milk buffer or PBS containing 1 % BSA (1 hour or usual time. Buffer formulations are given below). Alternatively, a tertiary labeling procedure may be used where the primary antibody is not biotinylated, but the second antibody is. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.
4. Rinse with PBS-Milk (3 X 1 min).
5. Incubate with FLUORONANOGOLD™-Streptavidin reagent diluted 1/5 - 1/20 in PBS- Milk with 1 % normal serum for 10 minutes to 1 hour at room temperature.
6. Rinse with PBS (3 X 1 min).
7. Postfix with 1 % glutaraldehyde in PBS at room temperature (3 mins).

8. Rinse in deionized water for (2 X 5 min).
9. If desired, contrast sections with uranyl acetate and/or lead citrate before examination.

Silver or gold enhancement may also be used to render the NANOGOLD® particles more easily visible (see below); this is recommended if stains such as uranyl acetate or lead citrate are applied. Silver or gold enhancement should be completed before these stains are applied.

PBS-Milk Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
5% Non-fat dried milk (final concentration)

*Optional, may reduce background:*

0.5 M NaCl
0.05% Tween 20
0.1% gelatin (high purity)

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

**SPECIAL CONSIDERATIONS FOR DIRECT VIEWING OF FLUORONANOGOLD™ IN THE ELECTRON MICROSCOPE**

For most work, silver enhancement is recommended to give a good signal in the electron microscope (see below). For particular applications, visualization of the FLUORONANOGOLD™ directly may be desirable. Generally this requires very thin samples and precludes the use of other stains.

FLUORONANOGOLD™ provides a much improved resolution and smaller probe size over other colloidal gold antibody products. However, because FLUORONANOGOLD™ is only 1.4 nm in diameter, it will not only be smaller, but will appear less intense than, for example, a 5 nm gold particle. With careful work, however, FLUORONANOGOLD™ may be seen directly through the binoculars of a standard EM even in 80 nm thin sections. However, achieving the high resolution necessary for this work may require new demands on your equipment and technique. Several suggestions 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 □l 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 must 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 1.4 nm size. This is the NANOGOLD®. For the 1:5 dilution suggested, there should be about 5 to 10 gold spots on the small viewing screen

used with the binoculars. Contrast and visibility of the gold clusters 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 makes the NANOGOLD® much more difficult to observe and not all of the golds are discernable. At 30,000 X (300,000 X with 10 X binoculars) NANOGOLD® particles are not visible. 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 enhancement to give particles easily seen at lower magnification.

## SILVER ENHANCEMENT OF FLUORONANOGOLD™-STREPTAVIDIN FOR EM

FLUORONANOGOLD™ will nucleate silver deposition resulting in a dense particle 2-80 nm in size or larger depending on development time. If specimens are to be embedded, silver enhancement is usually performed after embedding, although it may be done first. It 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 FLUORONANOGOLD™ reagents, low-temperature resins (eg Lowicryl) should be used and the specimens kept at or below room temperature until after silver development has been completed. Silver development is recommended for applications of FLUORONANOGOLD™ in which these stains are to be used, otherwise the FLUORONANOGOLD™ particles may be difficult to visualize against the stain.

Our LI SILVER silver enhancement system is convenient and not light sensitive, and suitable for all applications. Improved 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.[11]

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 the components immediately before use. NANOGOLD® will nucleate silver deposition resulting in a dense particle 2-20 nm in size or larger depending on development time. Use of nickel grids is sometimes preferred.

Fluorescence microscopy should be performed BEFORE silver enhancement. This is because the silver-enhanced gold particles can quench fluorescence.

The relevant procedure for labeling should be followed. Silver enhancement is then performed as follows:

1. Rinse with deionized water (2 X 5 mins).
2. OPTIONAL (may reduce background)[1]: Wash several times with 0.02 M sodium citrate buffer, pH 7.0.
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 min. 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. Alternatively, use of 0.1 % osmium tetroxide instead of 1 % has been found to give similar levels of staining while greatly reducing etching of the silver particles.

**NOTE**: Treatment with osmium tetroxide followed by uranyl acetate staining can lead to loss of the silver enhanced NANOGOLD® particles. This may be prevented by gold toning:[12]

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 OF FLUORONANOGOLD®-STREPTAVIDIN FOR EM

The small 1.4 nm NANOGOLD® particles may alternatively be enhanced (grown to a larger size) for better visibility using GoldEnhance™, which catalytically deposits gold around the NANOGOLD®, making a larger solid gold particle . Gold enhancement may be preferable to silver enhancement in some cases due to the different properties of GoldEnhance™: a) Gold is chemically more stable and is not depleted by osmium or uranyl stains; b) Gold has higher backscattering and is useful for SEM; c) GoldEnhance™ is light insensitive – it can be used in normal room lighting, and development followed in the light microscope; d) GoldEnhance™ may be used with physiological buffers, such as ones containing chloride, which precipitates silver enhancers. GoldEnhance™ follows a similar procedure to silver enhancement, but for specific directions, see those that accompany GoldEnhance™.

## IMMUNOBLOTTING

The basic procedure for gold immunoblotting has been described by Moeremans et al[13], 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. Fluorescence should be observed and recorded before silver enhancement, since silver-enhanced NANOGOLD® particles may obscure fluorescence. 10 mM biotin should be added to the third wash in step 6; this is because vacant biotin binding sites can quench the fluorescence of the fluorescein FLUORONANOGOLD™ label, and should therefore be blocked before observation. The procedure for immunoblots is as follows:

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. Incubate with biotinylated primary antibody according to usual procedure (usually 1 or 2 hours). Alternatively, a tertiary labeling procedure may be used where the primary antibody is not biotinylated, but the second antibody is. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.
4. Rinse with buffer 1 (3 X 10 mins).
5. Incubate with a 1/100 to 1/200 dilution of the Alexa Fluor®-FluoroNanogold™-Streptavidin reagent in buffer 2 for 2 hours at room temperature.
6. Rinse with buffer 3 (3 X 5 mins), then buffer 4 (2 X 5 mins).
7. OPTIONAL (may improve sensitivity): Postfix with glutaraldehyde, 1 % in buffer 4 (10 mins).
8. Rinse with deionized water (2 X 5 mins). Fluorescence may be observed at this point.
9. OPTIONAL (may reduce background): Rinse with 0.05 M EDTA at pH 4.5 (5 mins).
10. Develop with freshly mixed silver developer (LI SILVER™) for 15 – 30 minutes, or GoldEnhance™ LM/Blot for 10-20 minutes (or as directed in the instructions for these reagents). Development may be repeated (develop, rinse, develop with freshly mixed developer), to reduce background. Rinse thoroughly with deionized water between developments to remove all the reagent.
11. Rinse several times with deionized water.

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
5% non-fat dried milk
1% normal serum

*Optional, may reduce background:*

0.1% gelatin (Type B, approx. 60 bloom)
0.5 M NaCl
0.05% Tween 20

Buffer 3: 20 mM phosphate
150 mM NaCl
pH 7.4
5% non-fat dried milk
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, FLUORONANOGOLD-Streptavidin may be used as a tertiary labeled probe for a biotinylated secondary antibody, or as a probe for a directly biotinylated target. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.

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