TECHNICAL DATA SHEET

SPI Supplies
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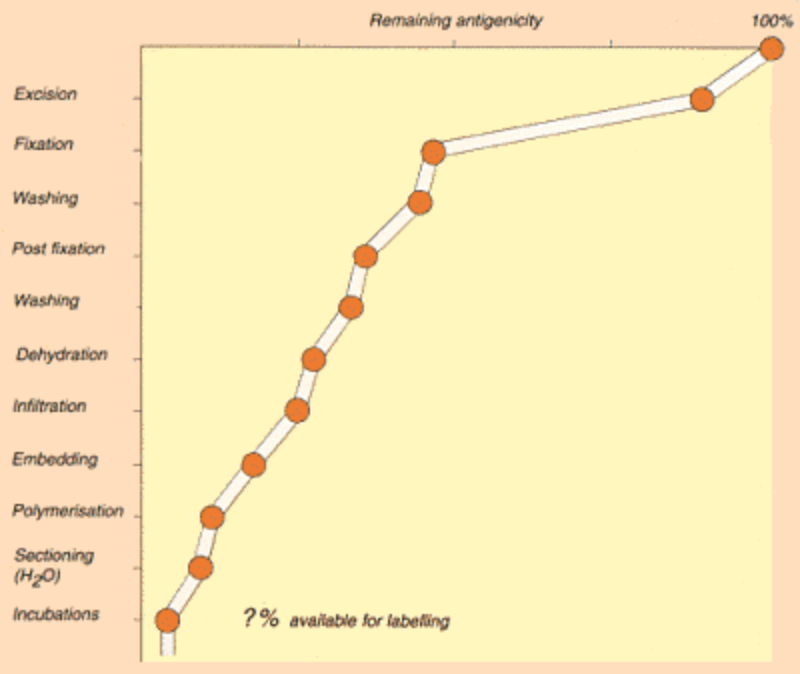
SPI-Mark™ Colloidal Gold Reagents

How to Get the Best from Immunogold labeling

All SPI-Mark gold and silver reagents come with detailed technical instructions for correct use in immunolabeling. Protocols for use are provided together with recommended dilutions, buffers, blocking reagents, etc. Each product is supplied with a detailed data sheet that provides information on specification and performance. Our Customer Service Group is always ready to give continuous advice for the use of our reagents with many different applications, including those that are out- of-the-ordinary.

The primary aim of all immunolabeling procedures is to obtain a maximum specific signal with a minimum non specific background. This is true for EM sections, LM sections, and immunoblots on membranes. The results obtained in any immunolabeling procedure are limited mainly by the specimen preparation and incubation procedures. References given here describe a number of optimum conditions and protocols for immunolabeling in LM, EM and Blotting applications. Although it is not possible to describe immunolabeling procedures for every possible application, extra protocols are available on request for basic approaches to each specimen type. These are described at the end of this page.

Specimen Preparation
Correct specimen preparation procedures are absolutely crucial for optimum labeling of antigens in cells and tissues. Methods commonly used for ultrastructural preservation in the EM or morphological preservation in the LM must usually be modified to ensure that antigens are not only retained but also available for labeling. This often involves compromise with the structure but careful selection of preparation methods can yield excellent combinations of structural detail and immunochemical labeling.



Potential loss of antigenicity during microscopical preparation procedures

For LM and EM studies cells and tissue sections may be studied by preembedding, post embedding, or cryotechniques. In addition whole cells may be examined as cytospins, cell smears, cells in culture, and cells in suspension 1,2,3,4.

It should be recognised that each step of the preparation procedure is likely to incur some antigenic loss by extraction or alteration or masking. Cumulatively these losses may greatly reduce the overall labeling unless each step is optimised. This can usually only be achieved empirically but some guidelines are described below.

a) Fixation

Cells and tissues may be fixed for subsequent examination or may sometimes be labelled in the unfixed state. Fixation of the tissue must also preserve antigenicity without compromise to structural characteristics. Fixatives either denature proteins by coagulation (eg acetone or methanol) or by forming additive cross linked compounds (eg aldehydes), or both. The resulting complexes inevitably differ from the unfixed proteins in both their chemical and antigenic profiles. Each tissue requires its own fixation protocol. For example, too much cross linking in a tissue with high protein density may mask many antigens. On the other hand a loose tissue with low protein content may disintegrate without adequate fixation and antigens may simply be washed out. According to the antibodies employed for antigen detection it may not be necessary to completely preserve the protein under investigation if at least the specific antigen is conserved. The types of fixatives employed are shown in the table below.

Types of Fixatives	Structure	Antigen Preservation	Suitability
Additive (cross linking)			
-Formaldehyde	++	++	Tissues
-Glutaraldehyde	+++	+	Tissues
Coagulative (precipitating)			
-Acetone	+	+	Cells
-Methanol	+	+	Cells
Mixed			
-Formal acetone	++	+	Cells
-Picric acid	++	+	Tissues

For many tissues the best compromise is a mixture of formaldehyde (eg 2- 4%) for rapid stabilising with low cross linking, and weak glutaraldehyde (eg 0.1%) for greater structural preservation. For cytological investigation a precipitating or coagulating fix such as acetone or methanol may be preferred. Formal acetone has also been commonly used for fixing cell preparations. In some cases for cell studies simple air drying may allow enough antigenic preservation for immunolabeling.

Post fixation for electron microscopy has mostly involved the use of osmium tetroxide in order to preserve membrane components and provide image contrast. The introduction of osmium into tissue is not always desirable, however, and more recently tannic acid has been suggested as an alternative[5].

Whatever method of fixation is selected it must serve the dual function of retaining the essential structural and antigenic components of the tissue without introducing any material which may interfere with the labeling. In some cases the introduction of heavy metals such as osmium or uranium into the tissue may cause increased non specific labeling and must be treated with caution.

b) Washing

Thorough washing of the tissues following fixation is extremely important. It may be necessary to wash for at least as long as the tissue has been fixed in order to remove excess aldehyde or other fixation residues which may cause non specific labeling. In some cases quenching the tissue with ammonium chloride is performed to neutralise aldehyde groups . The wash is best performed in a buffer having a tonicity similar to the natural tissue state.

c) Embedding

Tissues may be embedded in paraffin wax for LM, and resin for LM or EM studies. In either case the embedding should allow good preservation of the antigens without sacrifice of structural information. Generally there are two types of resin, epoxy resins which have an aromatic structure and are strongly cross linked, and acrylic resins which have lower cross linking. Epoxy

resins are hydrophobic whereas acrylic resins may be hydrophobic or hydrophilic. The best structural preservation and stability is provided by epoxy resins while the best immunolabeling is usually achieved with acrylic resins. This is because acrylic resins cut in such a way as to reveal the proteins at the section surface and they also wet more easily, thus giving greater accessibility to the antibodies during subsequent incubations.

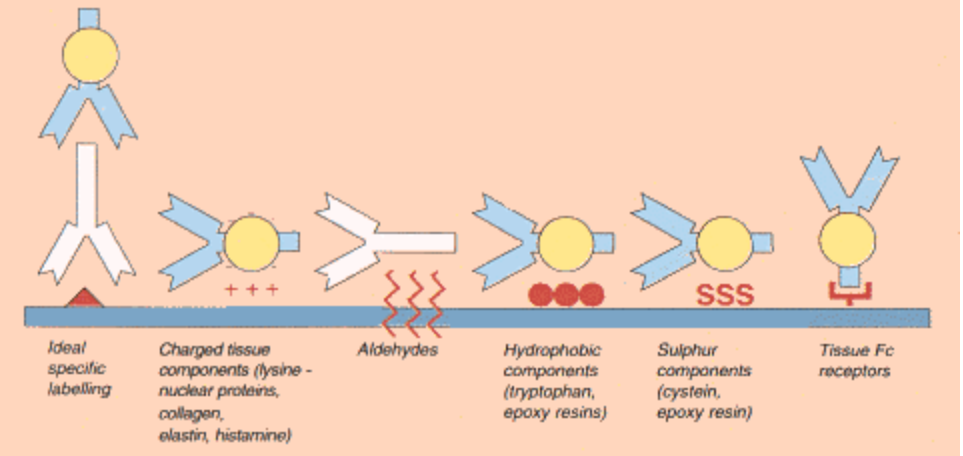
Resins for EM and LM preparation

Types of Resin	Cross Linking	Hydrophilicity	Structure/Stability	Antigenic Preservation/Access
Epoxy				
Araldite	High	Hydrophobic	+++	+
Epon	High	Hydrophobic	+++	+
Acrylic		Hydrophilic	++	+++
UNICRYL	-	Hydrophilic	++	++
Lowicryl®	-	Hydrophilic	++	++
LR White	-	Hydrophilic	++	++
LR Gold	-	+/-	+	+
Methacrylate		Hydrophilic		

For the optimum results a compromise must be reached and the usual choice is of a polar (hydrophilic) resin with moderate cross linking. A popular acrylic resin formulation is given by UNICRYL, which gives excellent immunolabeling characteristics together with a high degree of stability and structural preservation. UNICRYL, in common with many other acrylic resins, also allows the embedding procedure to be performed at low temperatures. This ensures that vital components are not extracted during dehydration and that no excessive temperature rise occurs during polymerisation which may damage the tissue antigens.

d)Blotting

For proteins blotted onto membranes by Western blotting or dot blotting it is important to ensure complete transfer of the proteins to the membrane. Membranes must then be blocked with neutral proteins or surfactants (eg BSA or Tween 20) to fill unoccupied sites and prevent non specific background labeling. The membranes are then incubated in a similar fashion to tissue sections. The complete protein band/spot pattern may first be revealed by immersing the membrane in a solution of PROTOGOLD which stains all proteins with colloidal gold, yielding a red stain. This gives an indication of the location of all protein bands. (See Protogold Section) A second identical membrane may then be incubated with the appropriate specific antibodies for identification of specific proteins immobilised on the membrane. An incubation of the membrane with gold labelled second antibodies will reveal the presence of specific protein bands by a visible red colour where the gold particles accumulate. In order to achieve the best signal with the least background thorough washing is important after each step.



Possible sources of non specific labeling

Incubations

In order to achieve the highest possible specific signal with the lowest possible non specific background it is important to be aware of all the factors in both specimen preparation and in subsequent incubations which can affect these results. The figure below indicates some of the most common sources of non specific background that can be identified by the use of controls and successfully eliminated. These factors apply for all types of tissues, whether for EM or LM, and for immunoblots on membranes.

a) Primary antibody

The primary antibody should be of high titre and of the highest specific purity to allow the greatest possible dilution. Cross reactivity must be low, especially with the sample tissue. A low quality primary antibody is the greatest cause of low specific signal and high background during the labeling procedure. The buffer composition and pH is important for the incubations. A normal TBS or PBS buffer is usually chosen with suitable additions to maintain low background (see below). The specimen must be washed thoroughly with buffer between incubations.

Typical dilution protocol for determining optimum signal to background labeling

Primary Antibody	Secondary Antibody(Gold)	Specific Signal	Non Specific Background
Series 1			
1/100	1/100	++++	++
1/1000	1/100	+++	+
1/10000	1/100	+	-
Series 2			
1000	1/50	++++	++
1/1000	1/100	+++	+
1/1000*	1/200*	+++	-

***Resulting optimum conditions for incubation.**

(b) Secondary antibody
A high quality second antibody is essential to label the primary specifically and with low background. SPI-Mark gold conjugates are affinity purified and of the highest quality. They are provided suspended in Tris or phosphate buffered saline for microscopical or immunoblotting use with sodium azide preservative and are stable for years under the correct storage conditions. This because our gold conjugates contain no free antibody, all antibodies being securely adsorbed at the surface of the gold. In addition the very high proportion of singlets ensures that clusters will not form in time during storage.

Their high titre and purity means that for incubations they may be used highly diluted (typically 1/100 - 1/400) in the typical incubating buffer shown below, so reducing non specific background whilst achieving a high signal intensity. Even at high dilutions (1/1000 or more) the conjugates are extremely stable and may be left for long term incubations.

(c) Dilutions
When establishing a new protocol it is necessary to determine the optimum concentrations of both the primary and the gold labelled secondary antibodies. This is done by first incubating separate sections with various dilutions of the primary antibody over an appropriate range of values, eg 1/100 to 1/10,000. The various primary incubations are then followed by second incubations, using a constant dilution such as 1/100 of the gold labelled secondary antibody. The dilution of primary antibody giving the optimum signal and background is thus determined. The procedure is then repeated, this time using the determined dilution of the primary as a constant, and incubating with a selected range of dilution values of the gold conjugate, eg 1/10 to 1/400. Observation of the second set of results will indicate the choice of dilution of conjugate that should be used in further experiments. The table below gives an illustration of a typical routine for these dilution series.

With both primary and secondary incubations a greater sensitivity may also be achieved by agitating the sample or flushing the reagents, so bringing fresh solution continuously to the target proteins at the tissue surface. If the antibody solutions are tolerant the incubations may also be performed at slightly elevated temperatures (up to 37°C).

d) Blocking
Non specific reactive sites on tissues and cell surfaces as well as unoccupied sites within blotting membranes may need blocking before antibodies are applied to the specimens. The causes of non specific labeling may arise from sources shown in the table below. Each background source will need its own blocking procedure either before or during the antibody incubations as shown in the table and as described in the BBI Technical Information Booklet supplied with each product. Blocking reagents are supplied by BBI and are listed separately in Blocking Reagents Section.

Typical sources of background in immunolabeling and appropriate remedies

Source of background Solution

1. Highly positively charged tissue components (eg lysine residues in histone proteins, collagen, elastin, histamine) attracting negatively charged gold particles (as described in Introduction to Gold labeling)
   a) Raise the pH of the incubation buffer to reduce positive charge
   b) Increase the salt content of the buffer to reduce the charge field of the gold particles
   c) Block the tissue with BSA before and during incubations
2. Non specific attraction of antibodies to tissue components through tissue aldehydes, fixative residues, or general non specific binding components
   a) Block the tissue with BSA before and during incubations
3. Hydrophobic tissue components (eg. tryptophan residues) attracting hydrophobic gold particles (as described in Introduction to Gold labeling)
   a) Add surfactant such as Tween 20 (eg 0.1-1%) to the incubation buffer
4. Sulphur containing tissue components (eg cystein residues, epoxy resins) attracting gold particles through dative bonding (as described Introduction to Gold labeling) Add increased BSA (eg 5%) to the gold buffer only to thoroughly coat the gold with sulphur before applying to the sections. (NB Do not add gelatin since it will increase the attraction of gold to the section).
5. Receptors in the tissues attracting second antibodies non specifically (eg gold labelled Goat anti-Rabbit)
   a) Block the tissue sections/membranes with normal serum (eg 10% normal goat serum) before primary incubation.*
   b) Add normal serum (eg 1% normal goat serum) to the incubation buffer to block the (goat) receptor sites.*
   c) Use F'ab gold conjugates (eg gold labelled Goat (F'ab) anti-Rabbit) (see Section 3)

A typical incubating buffer, suitable for both LM and EM incubations, which provides the components for eliminating the above sources of background may be as shown below:

Typical incubating buffer
PBS or TBS
+ 1% Normal serum* (eg goat)**
+ 0.1% Tween 20
+ 1% BSA + 0.1% sodium azide, adjusted to pH 8.2.

This buffer may be used for each step of the incubation protocol, followed by washing in water after the final step.

* Serum cannot be used in conjunction with Protein A, Protein G or Protein AG gold conjugates since these bind IgG molecules.

** Select serum corresponding to the host of the gold labelled antibody. In this example goat is selected because the conjugate being used is Goat anti-Rabbit.

e) Controls

In order to determine if a signal is genuine and to differentiate it from background labeling, both positive and negative controls must be used. These are simple to perform and should always be included in any staining protocol. Negative controls would typically include the following:

Omit the primary antibody
Use a non specific primary antibody of the same species
Absorb the primary antibody with antigen before incubation
Use a non specific second antibody

These negative controls will help identify the source of any non specific background. A good positive control using a specimen with high antigen content will test the whole labeling system. Full instructions for the use of controls are given in the BBI Technical Instruction Booklet supplied with each product.

For LM, EM and Blotting applications the correct use of silver enhancing procedures is also important to maximise the signal and to avoid non specific background. These procedures are described in our Silver Enhancing Section.

References:

1. Hayat MA (ed) (1989-91) "**Colloidal Gold. Principles. Methods and Applications". Vols 1-3**, Academic press, London.*
2. Bullock GR and Petrusz P (eds) (1982-90) "**Techniques in Immunocytochemistry" Vols 1, 2, 3, and 4, Academic Press, London.**
3. Baker JR (1970) "**Principles of Biological Microtechnique**", Methuen, London.
4. Lillie RD (1965), **Histopathologic Technique and practical Histochemistry, 3rd ed, McGraw Hill**, New York.
5. Berryman MA, et al (1992) Effects of tannic acid on antigenicity and membrane contrast in ultrastructural immunocytochemistry. **J Histochem Cytochem 40**, 6, 845-857

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