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QUANTOMIX™

QX-102
Applications Manual

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Table of Contents

List of Figures

List of Tables

Safety

Warnings

a. Appropriate safety protocols should be used when handling biological samples, especially human or primate derived cell lines and pathogenic microorganisms.

b. Many of the reagents used in fixation and staining protocols are toxic. Heavy metal stains (such as Uranium and Osmium) are long-term cumulative poisons. Observe the following cautionary rules when handling toxic materials:
   - Read carefully the labels and MSDS (Material Safety Data Sheet) and proceed accordingly.
   - In general, work with toxic materials should be preformed in a fume hood, wearing laboratory gloves, goggles and lab-coats.
   - All toxic waste should be disposed of according to the guidelines of local authorities.

Chapter 1: Introduction

Manual Scope and Contents

This manual provides protocols for preparing material and biological samples for imaging in QX-102 capsules.

The Applications Manual consists of the following chapters and appendices:

Chapter/ Appendix	Heading	Provides
1	Introduction	The manual scope and contents and introduction to QX-102 capsule applications.
2	Liquid Samples	General protocol for applying liquid samples.
3	Particles in Solution	Protocols for coating the capsule membrane for attaching particles.
4	Biological Applications	The theory and protocols for various biological applications, including sample handling application, fixation, staining and immunolabeling.
A	Glossary	The terms and abbreviations of the Applications Manual.
B	Troubleshooting	Troubleshooting instructions.

For detailed description of the QX-102 capsule technology components and general guidelines for handling the capsules and accessories, please refer to the QX-102 User Manual.

Technical Support

For technical support please contact tech@quantomix.com.

QX-102 Applications

The QX-102 capsules can be used for imaging various wet material and biological samples. The applications include liquid samples (emulsions, foods, oils, paints, inks, drugs etc.), pastes and foams (cosmetics, foods, etc.), particles in solutions, adherent and non-adherent cultured cells, and microorganisms. The contrast between water and fat is especially well visualized with WETSEM Technology, enabling analysis of fat structure and content in samples such as food and cosmetics. QX capsules can also be used for EDS (energy dispersive spectroscopy) analysis of samples using a SEM equipped with an EDS system. Samples in liquid form can be imaged directly simply by placing them inside the QX-102 capsule. Some material samples, such as beads in solutions, may require coating of the capsule membrane for proper attachment.
The QX-102 capsules are designed as miniature cell culture dishes, and are suitable for various cell biology applications. The sample preparation is comparable to light microscopy, and no drying, coating or embedding steps are required. Adherent and non-adherent cultured cells, as well as microorganisms, can be processed for imaging in the QX-102 capsules. The samples can be imaged either following an appropriate contrast enhancement staining, immunolabeling procedures, or without any treatment.

The sample preparation protocols provided in the Application Manual are divided into the following categories:

- Liquid Samples
- Particles in Solution
- Biological Applications

Chapter 2: Liquid Samples

The QX-102 capsule can be used for imaging any wet sample such as:

- Various foods
- Cosmetics, creams
- Emulsions
- Oil, grease
- Paint, ink

Liquid samples can be imaged directly, and usually no treatment of the sample or of the capsule membrane is required.

☞ To image liquid samples

1. Open the capsule.
2. Carefully apply 15 µl of liquid to the liquid dish.

Notes

a. Applying liquids is most conveniently done using standard lab pipettes.
b. Care should be taken not to touch the capsule membrane with the pipette tip.
c. When applying viscous solutions, creams, pastes, foams or similar samples, take care that no air bubbles are trapped between the sample and the capsule membrane.

3. Close the capsule.
4. Proceed to imaging according to guidelines provided in the QX-102 User Manual Chapter 3.

Chapter 3: Particles in Solutions

QX-102 capsules are suitable for imaging various particles such as macromolecules, beads and fibers in solutions. The sample part imaged with the QX capsules is the part proximal to the capsule membrane, and thus, for optimal imaging the sample must be in close contact with the capsule membrane. When the capsule is inverted in the SEM during imaging, some particles, depending on their size, weight and the composition of the solution they are suspended in, may not stay attached to the membrane. In these cases, treating the capsule membrane by coating agents may be useful.

Membrane Coating

The coating that provides best attachment depends on the type of specimen and the nature of the experiment. Coating protocols suitable for biological applications are provided in chapter 4. Below are protocols for coating the membrane with Poly-L-Lysine, a positively charged polymer, and with Poly (sodium-4-styrenesulfonate), a negatively charged polymer. These are suitable for attaching negatively or positively charged particles respectively. Other coating reagents may also be used, however, one should take into account that in order not to interfere with the imaging, the coating layer must be thin and should not contain electron-dense materials.

For coating procedures for specific applications, please see our website www.quantomix.com or consult tech@quantomix.com.

Poly-L-lysine coating

Poly-L-lysine is a positively charged polymer, to which negatively charged particles can attach.

The reagents required for Poly-L-lysine coating are:

- 0.1% w/v Poly-L-lysine in water (for example Sigma Cat. No. P8920)
- Distilled water

☞ To coat the capsule membrane with Poly-L-lysine

1. Apply 15 µl of 0.1% Poly-L-lysine solution to the liquid dish and incubate for one hour at room temperature. Longer incubation times, up to overnight, also give good results.
2. Remove the solution and rinse the liquid dish twice with distilled water.
3. Keep the liquid dish filled with water until applying the sample. Alternatively, remove the water and dry the liquid dishes for later use.

Poly (sodium-4-styrenesulfonate) coating (PSS)

PSS is a negatively charged polymer, to which positively charged particles can attach.

The reagents required for PSS coating are:

- 30% w/v Poly(sodium-4-styrenesulfonate) in water (for example Aldrich, Cat. No. 527483)
- Distilled water

☞ To coat the capsule membrane with PSS

1. Dilute the 30% stock solution to 0.3% w/v in distilled water
2. Apply 15µl of the diluted solution to the liquid dish and incubate for one hour at room temperature. Longer incubation times, up to overnight, also give good results.
3. Remove the solution and rinse the liquid dish twice with distilled water.
4. Keep the liquid dish filled with distilled water until applying the sample. Alternatively, remove the water and let the liquid dishes dry for a later use.

Sample Application

Particles can be attached to the coated membranes either by adsorption or by centrifugation. QX Imaging Buffer is the recommended imaging medium when applicable, since it is specially formulated to minimize damage to the samples by electron beam during imaging in the SEM. In applications where the particles can be firmly attached to the membrane and do not require to be suspended in a specific solution, we recommend changing the solution to QX-102 Imaging Buffer prior to imaging.

☞ To apply the sample on a coated membrane

1. Prepare a suspension of particles at the appropriate dilution.
2. Apply 15 µl of the suspension into the liquid dish and incubate for one hour at room temperature, or centrifuge at 500 g for five minutes in a centrifuge equipped with 96 well plate holders.
3. If applicable, wash with water several times and exchange to 15 µl QX-102 Imaging Buffer prior to imaging.
4. Seal the capsule.
5. Proceed to imaging according to guidelines provided in the QX-102 User Manual Chapter 3.

Chapter 4: Biological Applications

This chapter provides detailed protocols for preparing biological samples to be imaged using the QX-102 capsules. The samples can be imaged either untreated, fixed, stained or immunolabeled.

These protocols have been found to be widely applicable. However, since the results obtained depend on the type of application, the user should optimize the protocol for each application.

For latest protocol updates, please refer to our website: www.quantomix.com.

General Protocols

Many of the sample preparation steps, including cell attachment, fixation and immunolabeling, are similar to protocols generally used in cytological staining for light and fluorescent microscopy.

General staining protocols make use of heavy metals and are derived from protocols used in electron microscopy. The protocols are fast and easy to perform. No embedding or drying steps are needed and typical sample preparation periods are shorter than half a day, as shown in Figure 1.

The liquid dish can serve as a miniature cell culture dish, and cell attachment, spreading and growth on the electron-transparent capsule membrane have characteristics and requirements similar to standard cell culture dishes. Non-adherent cells may also be deposited on pre-coated membranes by adsorption or centrifugation.

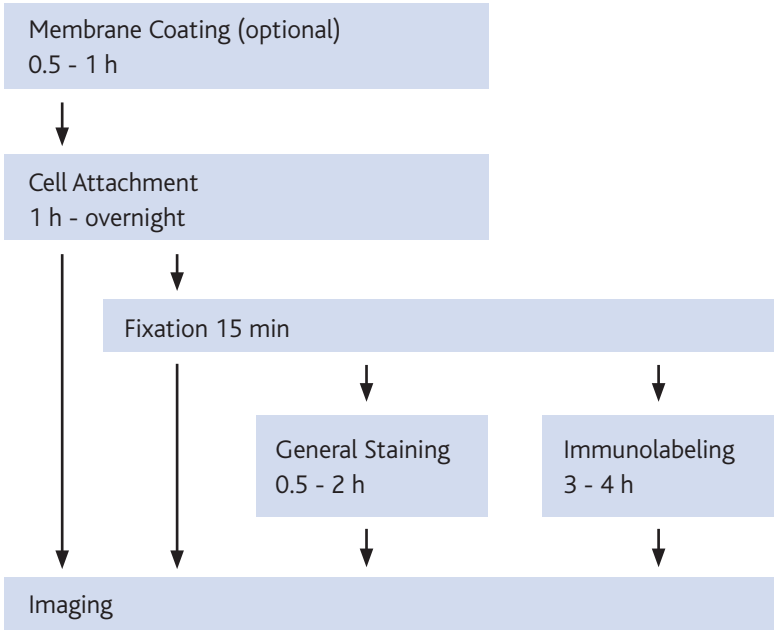

Figure 1: General Protocol Flowchart

After a specimen is applied to the capsule, it can be imaged in its native form or fixed. General staining using heavy metals can improve contrast. Labeling of specific proteins or cellular structures can be done using immunogold labeling protocols. Immunolabeled samples can also be counterstained to visualize cellular details. For examples of images obtained using the technology, refer to our website, www.quantomix.com.

The following protocols for preparing samples are described below:

- QX-102 membrane coating
- Sample application to the QX-102
- Handling native, non-treated samples
- Sample fixation
- General staining
- Immunogold labeling

QX-102 Membrane Coating Protocols

The capsule membrane supports growth of most cell types without additional treatments. However in most cases, to ensure optimal growth, pre-coating of the capsule membrane is recommended. The attachment factors that can be used vary from extra-cellular matrix components such as Fibronectin, Collagen or Gelatin to charged polymers such as Poly-L-lysine. The factors that provide best attachment depend on the type of specimen and the nature of the experiment. In choosing the most suitable coating protocol, one should take into account that the coating layer should be thin and should not contain electron-dense material.

The following coating protocols are provided below:

- Fibronectin
- Gelatin
- Poly-L-lysine

Fibronectin Coating

Fibronectin supports growth of many types of cells. If Fibronectin is not suitable, other extracellular matrix components, such as Collagen, Laminin or a mixture of ECM molecules may be used.

The reagents required for Fibronectin coating are:

- 0.1% Fibronectin solution (for example, Sigma F-1141)
- PBS

To coat the capsule membrane with Fibronectin

1. Dilute Fibronectin with PBS to a final concentration of 0.01%.
2. Apply 15 µl to the liquid dish and incubate for 30 minutes at room temperature.
3. Remove the solution and wash twice with PBS.
4. Wash twice with the appropriate growth medium.
5. Keep the liquid dish filled with the medium until seeding of cells.

Note

It is recommended to plate cells on fibronectin within one day from coating.

Gelatin Coating

The reagents required for Gelatin coating are:

- Gelatin (for example, Sigma Cat. No. G6144)
- Distilled water
- PBS

To coat the capsule membrane with Gelatin

1. Dissolve 0.1 % w/v Gelatin in distilled water at 37°C until no lumps are visible.
2. If desired, sterilize by filtration before coating or by UV irradiation of the coated liquid dishes.
3. Apply 15 µl to the liquid dish and incubate for one hour at room temperature.
4. Remove the solution and wash twice with PBS.
5. Keep the liquid dish filled with PBS until seeding of cells.

Notes

a. It is recommended to coat the capsule membrane on the day of use.
b. Higher concentration of Gelatin may improve the attachment for some specimens.

Poly-L-lysine Coating

Poly-L-lysine is a positively charged polymer, to which cells that carry overall negative charge can attach. Since it interferes with the growth and other physiological functions of some cells, it is not the first choice to be used as a coating agent for growing adherent cells in the QX-102 capsule. However, it is well suited for attachment of non-adherent cells or microorganisms.

The reagents required for Poly-L-lysine coating are:

- 0.1% w/v Poly-L-lysine in water (for example Sigma Cat. No. P8920)
- Distilled water

To coat the capsule membrane with Poly-L-lysine

1. Apply 15 µl of 0.1% Poly-L-lysine solution to the liquid dish and incubate for one hour at room temperature. Longer incubation times, up to overnight, also give good results.
2. Remove the solution and wash twice with distilled water.
3. Following the wash, keep the liquid dish filled with water until applying the sample. Alternatively, remove the water and dry the dishes in a sterile environment.

Sample Application Protocols

Cell Culture Growth

For adherent cells, the QX-102 capsule is used as a tissue culture dish for direct attachment and growth of cells. A large number of cell lines (for example, HeLa, CHO, A431 and NIH3T3) are grown in their regular growth medium and no special growth conditions are required. Assays or treatments of cells can be performed directly on the cells grown in the capsule. If desired, the cells can be grown and treated first in tissue culture dishes and then attached to the capsule membrane prior to imaging.

Cells grown in suspension can be attached to the capsule membrane by coating it with attachment factors such as Poly-L-lysine. Treatment of non-adherent cells, such as staining or labeling can be done in the capsule on attached cells, or alternatively, cells can be first treated in sample tubes and attached to the capsule membrane prior to imaging.

Cautions

a. For growing cells, it is important to use clean, sterile MP-10 multi-well plates.
b. Do not reuse multi-well plates that have been used for staining with toxic materials.

Adherent Cells

The reagents required for seeding adherent cells are:

- Normal growth medium
- Trypsin or non-enzymatic detachment reagent
- PBS

To seed an adherent cells culture

1. Coat the capsule membrane with Fibronectin or other suitable attachment factor prior to use. For details, refer to QX-102 Membrane Coating Protocols.
2. Detach the cells from their growth flask, for example by using Trypsin.
3. Wash with PBS or a growth medium and resuspend in a fresh growth medium. Count the cells.
4. Dilute the cells so that 15 µl of seeding medium in the dish contains the desired amount of cells. For example, use the guidelines supplied in Table 1.

Note

Usually, seeding 1000 to 2000 cells in each dish renders a sub-confluent density of adherent cells after overnight growth. The optimal cell density should be adjusted according to the application.

Table 1: Guidelines for Cell Dilution

Cells/Dish	Cell Concentration	Seeding Volume
1000	6.6 x 10^4/ml	15 µl
1500	1 x 10^5/ml	15 µl
2000	1.3 x 10^5/ml	15 µl

5. Inoculate 15 µl of the cell suspension on the capsule membrane.
6. Fill the reservoirs along the edges of the MP-10 multi-well plate with distilled water.

Note

To maintain a humid atmosphere and avoid sample drying, it is important to fill the reservoirs along the edges of the MP-10 multi-well plate. For detailed instructions, refer to QX-102 User Manual.

7. Incubate in the normal growth environment (for example, an incubator with a humid, 5% CO_2 atmosphere) overnight or until the cells are attached.

Typical protocol outline for growing adherent cells, found to be widely applicable to many cell types, is provided below:

Typical protocol outline for applying a sample of adherent cells

1. Coat with 0.01% Fibronectin for 30 minutes at room temperature.
2. Wash twice with PBS.
3. Wash twice with culture medium.
4. Seed 2000 cells in each capsule.
5. Fill each lateral reservoir of the multi-well plate with 200 µl of water.
6. Close the lid and incubate at 37°C until the cells are attached (usually overnight).

Cells in Suspension and Microorganisms

QX-102 capsules can be used for imaging cells and microorganisms grown in suspension, such as lymphocytes, bacteria or protozoa. Since these organisms usually do not attach directly onto the capsule membrane, coating is required. Preferred attachment protocols make use of Poly-L-lysine or Gelatin coating. Cells are attached to the coated capsule membranes by incubating or by centrifuging. The specimens can be imaged, either in their unstained form or fixed and stained according to the protocols provided below.

The suspension of cells can be maintained in a culture medium or buffer and may be fixed to the membrane before application. The dilution factor of the suspension depends on the type of organism and culture used and should be determined experimentally. Labeling of specific antigens can be performed using immunolabeling, either before or after attachment to the capsule membrane.

The following protocol is applicable for attaching cells in suspension, such as bacteria or other unicellular organisms to the capsule membrane.

For application of a suspension of cells or microorganisms

1. Coat the capsule membrane with either Poly-L-lysine or Gelatin. For details, refer to QX-102 Membrane Coating Protocols.
2. Wash the membrane with PBS and keep it filled with PBS until use or alternatively, wash with distilled water and dry overnight.
3. Prepare a suspension of cells, microorganisms or particles at the appropriate dilution (for example, for the E.coli, 1:100 dilution of an over-night culture renders the appropriate number of bacteria in a capsule).
4. Apply 15 µl of the diluted culture into the liquid dish and incubate for one hour at room temperature, or centrifuge at 500 g for five minutes at room temperature in a centrifuge equipped with 96 well plate holders.

Caution

To avoid interference with cell adsorption onto the membrane, it is recommended not to include Serum or Formaldehyde in the suspension.

5. Optional: To stabilize the binding after cell adsorption, fix the cells briefly with 4% Formaldehyde/PBS for 10 minutes or 2.5% Glutaraldehyde/PBS for five minutes.
6. Wash with PBS, or water if the cells are fixed, and proceed to staining or imaging.

Handling Native, Non-treated Samples

The wet environment of the QX-102 capsule enables visualizing cells in their native, non-fixed conditions. Even in unstained samples, the differences between the various constituents of the cell often generate sufficient contrast to distinguish some level of details. High-density materials such as salts, phosphorous or iron concentrated in different regions of the cells may improve the contrast.

The level of radiation absorbed by the cells during imaging at high magnification is expected to affect their viability. On short time imaging, no obvious structural damage is apparent and several repeated scans of cells give the same images. Thus, short time imaging of living cells may be possible. Live cells can be attached to the capsule according to the protocols provided above and imaged directly in growth medium or in PBS.

Fixation Protocols

Most of the general staining and immunolabeling protocols are carried out after fixation of the specimen. The purposes of the fixation are:

- To preserve the cellular structures as close to the living state as possible.
- To protect the sample from morphological alteration and damage during the subsequent treatments.

A wide range of fixatives commonly used in cytological, immunostaining and electron microscopy studies can be applied to the QX-102 capsule samples. Since no fixative preserves all the cellular structures, an appropriate choice of fixatives depends on the specimen, and the cellular details of interest. For immunolabeling, the choice of the most suitable fixation protocol is also affected by the nature of the antigen and antibody.

Warning

Since all fixatives are toxic to some extent, all work should be performed in a fume hood using gloves and protective clothing. Handling and waste disposal should be according to guidelines of the local authorities.

Aldehydes

Fixation in protein-crosslinking Aldehyde reagents, such as Paraformaldehyde or Glutaraldehyde, is usually the first choice. Fixation with Glutaraldehyde is permanent, whereas Paraformaldehyde may be partially reversed during long incubation times with solutions. Glutaraldehyde penetrates slower than Paraformaldehyde and thus requires longer fixation times.
The following two sections provide standard fixation protocols for Paraformaldehyde and Glutaraldehyde. The optimal concentration and time of

fixation depend on the application and should be determined experimentally. Samples fixed for subsequent immunolabeling require special precautions to avoid damaging the relevant epitopes. For some applications, a mixture of Paraformaldehyde and Glutaraldehyde may render improved results.

Glutaraldehyde Fixation

The reagents for Glutaraldehyde fixation are:

- 25% Glutaraldehyde solution EM grade (for example, Agar Cat. No. R1020)
- PBS

To fix the specimen with Glutaraldehyde

1. Dilute the Glutaraldehyde stock solution to a 2% final concentration in PBS.
2. Wash the sample four times with PBS.
3. Incubate with 2% Glutaraldehyde/PBS at room temperature for 30 minutes.
4. Wash four times with PBS.
5. Proceed to staining or labeling reaction.

Note

For some applications, better results can be obtained with lower concentrations of Glutaraldehyde (0.2% to 1%) and longer incubation periods.

Paraformaldehyde Fixation

The reagents required for Paraformaldehyde fixation are:

- Paraformaldehyde, EM grade (for example, a 16% solution, Electron Microscopy Sciences, Cat. No. 15710)
- PBS

☞ To fix the specimen with Paraformaldehyde

1. Prepare 4% Paraformaldehyde solution in PBS.
2. Wash the sample four times in PBS.
3. Fix with 4% Paraformaldehyde/PBS at room temperature for 15 minutes.
4. Wash four times with PBS.
5. Proceed to staining or labeling reaction.

Note

For some staining and labeling protocols, 2% Paraformaldehyde may give better results.

Organic Solvents

Organic solvents such as alcohols are also commonly used fixatives. Since these reagents dehydrate the cell and remove lipids, they can destroy some of the cell architecture. These fixatives may be preferred if immunolabeling is desired, since in some cases they may preserve target epitopes better than Aldehyde fixatives.

Caution

Please note that the QX-102 capsule is not compatible with Acetone and Toluene. For other organic solvent please consult with tech@quantomix.com.

Methanol Fixation

The reagents for Methanol fixation are:

- Methanol cooled to –20°C
- PBS

☞ **To fix the specimen with Methanol**

1. Wash the sample four times with PBS at room temperature.
2. Change to pre-cooled (-20°C) 100% Methanol.
3. Incubate for five minutes at 4°C or at room temperature.
4. Wash four times with PBS at room temperature.
5. Proceed to staining or labeling reaction.

General Staining Protocols

The imaging contrast in QX-102 capsules is created from variations in atomic numbers of the sample constituents. Thus, heavy metal stains, such as Uranium and Osmium compounds, are best suited for improving the general contrast of the biological samples, whose main constituents are Carbon, Hydrogen, Oxygen and Nitrogen. Heavy metal stains attach, generally non-specifically, to cellular constituents. However, different affinities to various molecules enable visualization of some cellular structures.

The following staining materials are described below:

- Uranyl Acetate
- Phosphotungstic Acid (PTA)
- Osmium Tetroxide

Uranyl Acetate Staining

Uranium is the heaviest metal used in staining and can be used as a general contrast agent. Uranyl Acetate binds to nucleic acids, to proteins and to membranous structures.

Warning

Uranium compounds are toxic and radioactive. Contact your safety officer or local authorities for appropriate handling and disposal protocols.

The reagents required for Uranyl Acetate staining are:

- Uranyl Acetate (5% stock, pH 3.5 with HCl, kept at 4°C in the dark)
- Tannic Acid (2% stock in water)
- 4% PFA in PBS

- PBS
- Distilled water
- 0.45 µm syringe filters

To stain the specimen with Uranyl Acetate

1. Before starting, prepare fresh 1% Tannic Acid in distilled water and acidic 0.5% Uranyl Acetate (diluted in water from stock and filtered through 0.45 µm syringe filters).

Notes

a. Since Uranyl precipitates in the presence of Phosphate, samples must be rinsed thoroughly to remove traces of Phosphates before Uranyl staining.
b. Perform all the following steps at room temperature.

2. Wash the sample four times with PBS.
3. Fix with 4% Paraformaldehyde/PBS for 15 minutes. For details, refer to Fixing Protocols.
4. Wash the sample four times with PBS.
5. Wash four times, five minutes each wash, with distilled water.
6. Incubate with 1% Tannic Acid for 5 minutes.
7. Wash twice, five minutes each wash, with distilled water.
8. Incubate with 0.5 % Uranyl Acetate for 30 minutes.

Note

The optimal concentration and incubation time may vary depending on the specimen.

9. Wash twice, five minutes each wash, with distilled water.

10. Prepare the sample for imaging according to Preparing the Samples for Imaging Section (Page 40).

PTA (Phosphotungstic Acid) Staining

Phosphotungstic acid (PTA) is an anionic stain. PTA positively stains charged structures such as basic proteins associated with nuclear DNA and nucleoli and intensely stains mitochondrial matrix.

The reagents required:

- Phosphotungstic acid (for example Sigma Cat. No. P4006), 2% stock solution in double distilled water, pH 1.5.

Warning

PTA solution is acidic. Appropriate personal protective clothing should be used.

- Double distilled water
- 2% Glutaraldehyde in PBS

Notes

The stock can be stored at room temperature for approximately one month.

Procedure:

1. Wash the cells four times with PBS.
2. Fix cells with 2% Glutaraldehyde in PBS for 30 minutes.

Notes

Fixation can also be done with a combination of 2% Paraformaldehyde and 1% Glutaraldehyde in PBS for 30 minutes.

3. Wash three times with PBS.
4. Wash three times with double distilled water.
5. Incubate the sample with 2% PTA for 30 minutes.
6. Wash five times with double distilled water.
7. Prepare the samples for imaging according to Preparing the Samples for Imaging (Page 40).

Osmium Tetroxide Staining

Osmium Tetroxide is traditionally used in electron microscopy both as a fixative and heavy metal stain. Osmium Tetroxide is a good fixative and excellent stain for lipids in membranous structures and vesicles. The most prominent staining in adherent human cells (HeLa) is seen on lipid droplets (see www.quantomix.com, gallery). Some intracellular structures are also visualized. Visualized cellular structures depend on the fixation protocols; in Glutaraldehyde fixation nucleoli are visible, but overall nuclear staining is weak. In Paraformaldehyde fixation nuclear staining becomes more prominent, but some intracellular structures are lost. As a first choice, fixating with a combination of Glutaraldehyde and Paraformaldehyde is recommended.

Warning

Since OsO_4 is toxic and volatile, all work should be performed in a fume hood using gloves and protective clothing. Handling and waste disposal should be done according to the guidelines of the local authorities.

The reagents for Osmium Tetroxide staining are:

- 4% OsO_4 (for example, Sigma Cat. No. 75632)
- 2% Paraformaldehyde/0.1% Glutaraldehyde in PBS
- Distilled water
- PBS

To stain cells with Osmium Tetroxide

Note

All the following steps should be performed at room temperature.

1. Wash the cells four times with PBS.
2. Fix with 2% Paraformaldehyde/0.1% Glutaraldehyde/PBS for 30 minutes.
3. Wash four times with PBS.
4. Wash four times with distilled water.
5. Prepare 0.1% OsO_4 solution by diluting the 4% stock solution in distilled water.
6. Incubate the sample with 0.1% OsO_4 for 30 minutes.

Note

The optimal incubation time may vary between samples and should be experimentally determined.

7. Wash four times with distilled water.
8. Prepare the samples for imaging according to Preparing the Samples for Imaging Section (Page 40).

Immunogold Labeling Protocols

Colloidal gold particles are readily visualized in the QX-102 capsules. Gold beads conjugated to a variety of molecules, such as Protein A, Immunoglobulins and Streptavidin, are commercially available and can be used to immunolabel specific antigens. Since immunogold labeling combined with QX-102 capsules allows visualization of single gold particles attached to single molecules, the following unique advantages are offered:

- Imaging receptors in the context of the cell membrane
- Extremely low detection limit, allowing detection at single label sensitivity
- Ultra-fine localization and distribution studies are possible due to the 10 nm resolution range
- Labeling quantification by counting the individual particles

Immunolabeling protocols consist of four main steps:

- Fixation
- Blocking
- Antibody binding
- Detection with gold conjugate

For the immunolabeling protocol flowchart, refer to Figure 2.

Surface antigens can be labeled on live or fixed cells. Intracellular antigens can be labeled on fixed, permeabilized cells. Since fixation protocols can mask or change some epitopes, the optimal fixation protocol for each antibody should be experimentally determined.

In addition, optimal blocking for non-specific backgrounds, concentrations and incubation time with the primary antibody depend on the antigen and antibody in question. Since in some cases, specific incubation and wash buffers are required to avoid non-specific binding, there is no standard protocol that works for all labeling reactions. Optimal conditions may be established based on prior experience with the particular antibody and antigen or on preliminary experiments using immuno-fluorescence.

This section provides the outline and general guidelines, which should be modified according to the user-defined optimal conditions.

Commercial conjugated gold particles are available in different sizes, varying from 0.8 nm to 100 nm. Smaller particles have the advantage of more efficient labeling, but silver enhancement is required for their visualization. Larger particles can be visualized without further treatment. To obtain optimal conditions for each gold conjugate, refer to the manufacturer's recommendations.

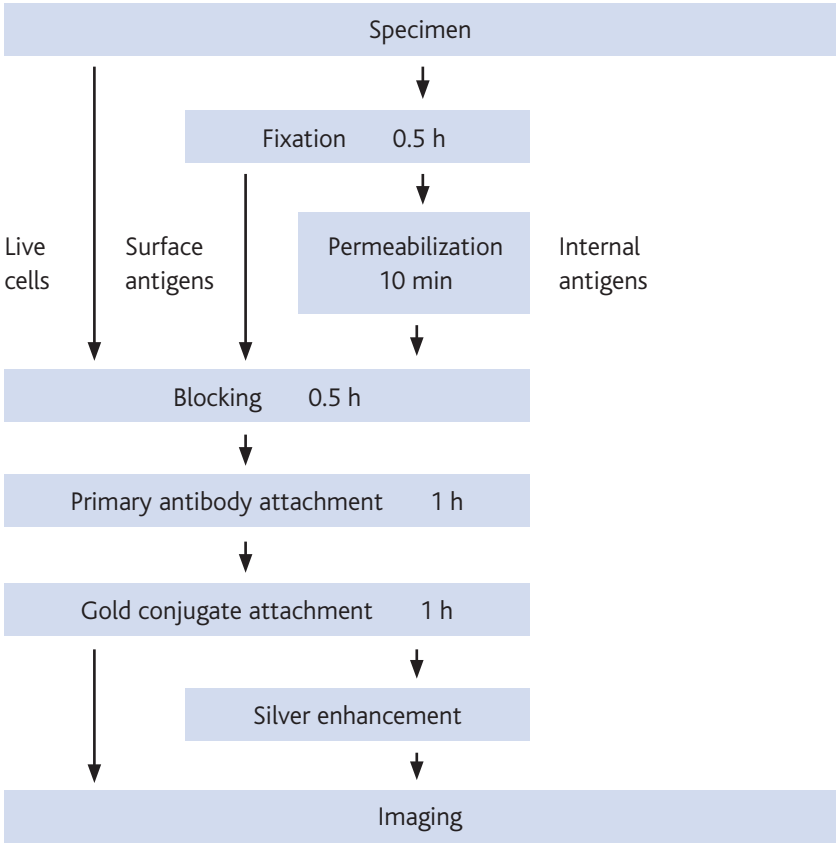

Figure 2: Immunolabeling Protocol Flowchart

To assess the labeling characteristics, the reactions should be compared using appropriate controls. Control reaction, in which the primary antibody has been omitted, should always be included.

Immunogold Labeling Reaction

The reagents required for the immunolabeling reaction are:

- PBS
- Fixative
- 0.2% Triton® X-100 in PBS (for intracellular antigens)
- Blocking agent (BSA, normal Serum or other)
- Primary antibody
- Gold particle conjugate
- Distilled water
- Silver staining kit (for example, AURION R-GENT SE-EM, Cat. No. 500.033)

To immunogold label a sample in a QX-102 capsule

1. Coat the membrane with Fibronectin or another appropriate attachment factor.
2. Attach the cells to the membrane.

Note

All the following steps should be performed at room temperature.

3. Wash four times with PBS.
4. Fix the cells, referring to Fixation Protocols. Omit the fixation step when live cells are labeled.
5. Wash four times with PBS.
   For intracellular antigens:

a. For Paraformaldehyde or Glutaraldehyde fixed cells, permeabilize the cells by incubating with 0.2% Triton® X-100/PBS for 10 minutes. If Methanol fixation is used, no additional permeabilization is required.
b. Wash twice with PBS - 5 minutes each wash.

6. To avoid non-specific background, incubate with a blocking solution, such as 1% BSA and 5% normal Serum from the species of the secondary antibody in PBS for 30 minutes.
7. Incubate with primary antibody in 1% BSA in PBS.
In parallel, carry out the control reaction without the primary antibody.

Notes

a. Carry out and test serial dilutions to determine the optimal concentration of the antibody.
b. Incubation periods of 30 to 60 minutes at room temperature usually render good results.
c. For some antibodies, labeling can be improved by incubating at 37°C or by longer incubation periods (several hours to overnight) at 4°C.

8. Wash four times with PBS, with BSA 1%.

Notes

a. In case of background problems, a mild detergent such as 0.05% to 0.1% Tween® 20 can be added to the wash buffer.
b. For surface staining or labering, do not use detergents.

9. Incubate with the gold-labeled secondary reagent (gold conjugated secondary antibody or Protein A or G) in protein containing solution, such as 1% BSA or 5% normal Serum.

Notes

For optimal dilution and conditions, refer to the manufacturer's recommendations.

10. To remove unbound antibodies, wash four times with PBS.
11. For gold beads smaller than 30 nm, perform silver enhancement.

Note

The AURION R-GENT SE-EM kit is recommended. However, other comparable kits are also available.

12. Wash six to ten times with distilled water.
13. Prepare the sample for imaging according to Preparing the Samples for Imaging Section (Page 40).

Preparing the Samples for Imaging

The QX Imaging Buffer is specially formulated to minimize the damage to the samples by the electron beam during imaging in SEM and should be used when ever applicable, especially for biological samples. Prolonged storage of the specimens in the imaging buffer is not recommended. For imaging live specimens, growth buffer of the samples or PBS should be used instead of the QX Imaging Buffer.

Caution

Solutions containing DMSO are not suitable for imaging in QX-102 capsules.

To prepare the samples for SEM-imaging

1. When the sample is ready, exchange the liquid in the liquid dish to 15 µl QX-102 Imaging Buffer.
2. Seal the capsule.

Notes

For optimal imaging results, we recommend to proceed directly to imaging.

If required, the closed samples can be stored at 4°C for short periods. Refrigerated capsules should be equilibrated to room temperature before inserting them to the SEM.

3. Place the QX-102 capsule in the SEM with the capsule membrane facing upwards and proceed to imaging according to the guidelines provided in the QX-102 User Manual Chapter 3.

Appendix A: Glossary

Term	Description
SEM	Scanning electron microscope
MP-10	Multi-well plate, a sterile, transparent holder for parallel handling of up to 24 individual QX-102 capsules, serving as a cell culture apparatus and holds the capsules during various manipulations
MA-4	Multi-well aspirator, a parallel drainage system designed to safely aspirate liquids from the QX-102 capsules without damaging the capsule's membrane
QX-102	Capsule used for SEM-imaging of a variety of liquids and wet samples
Liquid Dish	QX-102 capsule base designed as miniature cell culture dish for applying samples
Sealing Stub	Part of QX-102 capsule used for sealing the capsule and for holding the capsule in SEM
Calibration Capsule	QX-capsule with control sample used for optimization of imaging conditions
QX Imaging Buffer	Buffer optimized for imaging samples in SEM with QX-102 capsules
BSA	Bovine Serum Albumin
BSED	Back-scattered electrons detector
BSE	Back-scattered electrons
PBS	Phosphate-Buffered Saline
ECM	Extracellular Matrix
PFA	Paraformaldehyde
GA	Glutaraldehyde
w/v	Weight to volume

Appendix B: Troubleshooting Instructions

Phase	Problem	Possible Cause	Solution
Liquid Handling	Liquid is leaking out of the liquid dish.	The capsule membrane has been damaged.	a. Avoid touching the capsule membrane at any time.
			b. Always place the capsules in the MP-10 multi-well plate.
			c. Do not use means other than the MA-4 multi-well aspirator for aspirating liquids.
	Sample particles/cells are detached during liquid handling, especially from the center of the liquid dish.	Vacuum used for liquid handling is too strong.	Use weaker vacuum for liquid handling. See Instructions for MA-4.
		Sample particles/ cells are not attached well.	Increase the concentration of the attachment factor or try other factors.
Cell Growth	The cells do not attach to the capsule membrane.	The attachment factor in use does not support cell growth.	Use other attachment factors.
	The cells do not grow well.	The growth conditions are not optimal. Some cells may require specific conditions for growth in a QX-102 capsule.	Adjust the density of the cells or the incubation period.
		Traces of toxic materials spilled onto the MP-10 multi-well plate affect the cell growth.	An MP-10 multi-well plate that has been used for staining with toxic reagents *should not* be used for cell growth.

Phase	Problem	Possible Cause	Solution
Cell Staining	The cells look damaged after the staining/ labeling procedures.	Samples have dried while being handled.	Do not leave liquid dishes with low liquid levels for prolonged periods of time.
Imaging	No signal is observed.	The sample is not in contact with the capsule membrane.	For protocols of sample attachment, see Chapter 3 (Particles in Solution) or Chapter 4 (Biological Applications).
	The image is not clear.	There is no sufficient contrast between constituents of the sample.	The sample may require contrast enhancement, such as heavy metal staining. For staining of biological samples, see Chapter 4.

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