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Sample Preparation for Fluorescence Microscopy: An Introduction

Concepts and Tips for Better Fixed Sample Imaging Results

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Products: Cytation 3 and Cytation 5

Multiple processing steps are required to prepare tissue culture cells for fluorescence microscopy. Experiments are generally classified as being either live or fixed cell microscopy. Fluorescence microscopy of live cells uses either genetically encoded fluorescent proteins (e.g. GFP, mcherry, YFP, RFP, etc.) or cell membrane-permeable, non-toxic fluorescent stains. Fluorescence microscopy of fixed cells uses a fixative agent that renders the cells dead, but maintains cellular structure, allowing the use of specific antibodies and dyes to investigate cell morphology and structure. Appropriate sample preparation is necessary to ensure high quality images are captured. Here we describe a number of concepts and considerations regarding the sample preparation process that can assist with automated digital fluorescence microscopy of fixed cells.



Cell Fixation

The goal of fixation is to maintain cellular structure as much as possible to that of the native or unfixed state during the processing steps and subsequent imaging. There are a number of fixation methods suitable for fluorescence microscopy that fall into two basic categories: aldehyde fixatives and alcohol fixatives. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking aldehyde reagents form intermolecular bridges, normally through free amino groups, creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require a permeabilization step to allow the antibody access to the specimen. Fixation with both methods may denature protein antigens, and for this reason, antibodies prepared against denatured proteins may be more useful for cell staining. As each method has its advantages and disadvantages and because different fixative methods can destroy or mask different epitopes, several different methods may need to be tested for optimal results.

Aldehydes

Aldehyde fixatives cross link proteins and generally do an excellent job of preserving cell morphology. While they are generally slower acting than organic based fixatives, 4% formaldehyde for 10 minutes at room temperature is a good starting point. Glutaraldehyde will also cross link proteins, but results in significant autofluorescence and generally should be avoided or used in low concentration in conjunction with formaldehyde. Note that glutaraldehyde is the preferred fixative of choice for electron microscopy.

Formaldehyde vs. Paraformaldehyde vs. Formalin

Paraformaldehyde is a polymer of formaldehyde. It exists as a dry powder suitable for storage, and needs to be broken down into its monomeric component formaldehyde. This is accomplished by heating under basic conditions until it becomes solubilized. Commercial formaldehyde solutions generally referred to as formalin, often contain methanol to prevent re-polymerization into paraformaldehyde. A saturated formalin solution has formaldehyde content in water or buffer of 37-40% and is sometimes referred to as "100% formalin". Therefore using 10% formalin (1:10 dilution) is equal to using 3.7-4% formaldehyde for fixation. Since "100% formalin" contains up to 15% of methanol as a stabilizer, it can have a significant impact on fixation. This is mainly the result of membrane permeabilization by methanol, which results in the interference in the staining of membrane bound proteins.

Prepare a fresh 4% formaldehyde solution by dissolving paraformaldehyde powder into PBS (PH 7.4) using a stirring hot plate with the heater set to a medium setting until the liquid reaches approximately 60 °C. As the paraformaldehyde breaks down to formaldehyde it will dissolve. Once the solution is completely dissolved (approx 30 min), the solution is quickly chilled on ice back to room temperature prior to use. Typically, tissue culture cells are fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS followed by 2-3 washes with PBS to remove excess formaldehyde and stop the fixing reaction.

Alcohols

Organic solvents such as methanol rapidly precipitate proteins, maintaining structure when doing so. While the cellular cytoskeletal structure is maintained with methanol fixation, small molecules within will be lost during the subsequent processing steps because they are not precipitated. Because methanol precipitates proteins never use this method if genetically encoded fluorescent proteins are to be imaged or detected - activity from these proteins will be abolished with methanol fixation. Methanol is used cold (-20 °C) for 10-20 minutes. Using a combination of methanol and acetone (1:1) can sometimes improve results. Methanol is best for preserving structure while acetone improves permeabilization. Following fixation samples are washed with PBS 2-3 times to remove alcohol and rehydrate the specimen.

Buffer Selection

Another important consideration of a fixation protocol is the buffer selection. Ideally the buffer is isotonic in nature so as to not disrupt cellular structure, while maintaining a pH as close to physiologic as possible. The most frequently used buffer is phosphate buffered saline (PBS). When using aldehydes as fixatives avoid using amine-containing buffers, such as Tris, as they will react with the fixative.

Permeabilization

Fixation with the use of cross linking agents does not effectively diminish cell membrane structure. Therefore, formaldehyde fixation requires the cells to be permeabilized to allow antibodies access into the interior of cells. The large size and ionic nature of antibody proteins precludes them from gaining access without membrane disruption (Figure 1-2). Alcohol and acetone based fixation generally does not require this step.

Digitonin, Leucoperm and Saponin are relatively mild detergents that generate large enough pores for antibodies to pass through without completely dissolving the plasma membrane. These are suitable for antigens in the cytoplasm or the cytoplasmic face of the plasma membrane (Figure 1). Saponin acts by dissolving cholesterol present in the plasma membrane. When used at low concentrations, internal membranes remain intact so it is useful for labeling smaller molecules that exist in a soluble state within the cytoplasm. It should be prepared as a stock in DMSO, and is typically used at 0.5 to 1 mg/mL at room temperature for 10 to 30 minutes.

Triton X-100 is probably the most commonly used permeabilization agent for immunofluorescent staining. This detergent efficiently dissolves cellular membranes without disturbing protein-protein interactions (Figure 1). Triton is usually used at concentrations ranging from 0.1 to 1% for permeabilization. We typically use Triton X-100 or NP-40 at 0.1% in PBS at room temperature for 10 minutes. Besides cell membrane permeabilization, these detergents will partially dissolve the nuclear membrane making them suitable for nuclear antigen staining.

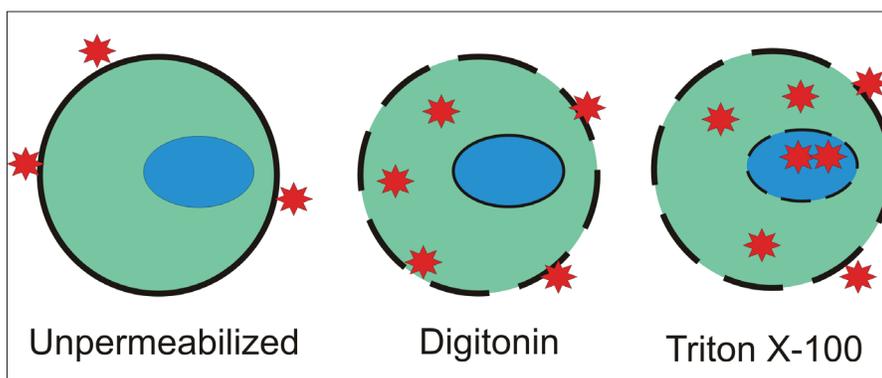


Figure 1. Antibody Accessibility with Unpermeabilized and Digitonin or Triton X-100 Permeabilized Cells. Antibodies can only access the exterior of aldehyde fixed unpermeabilized cells, while mild agents such as digitonin will permeabilize the plasma membrane allowing access to cytoplasmic interior, but not interior membrane bound organelles such as the nucleus of mitochondria. Stronger nonionic detergents, such as Triton X-100, permeabilize both the plasma membrane and interior membranes, allowing full access while still preserving cell structure.

SDS is an anionic detergent that is commonly used to denature proteins and provide them with a large negative charge for electrophoresis. It is useful as a permeabilizing agent to induce slight denaturation of fixed cells in order to reveal epitopes which may normally be masked from an antibody. It may extract small, poorly cross-linked proteins from fixed specimens, and should not be used on samples fixed by precipitation (e.g. methanol).

Blocking

When using antibodies to stain cellular objects in specimens, it is necessary to “block” the sample in order to reduce non-specific binding. Non-specific binding may occur for several reasons: unreacted fixative aldehydes may crosslink antibodies to inappropriate structures; structures within the samples may ‘trap’ antibodies; or, if using polyclonal antibodies, low affinity IgG molecules may bind to inappropriate structures. These potential issues may be prevented by treating the specimens with a protein solution that will compete for non-specific binding sites prior to staining with antibodies. Commonly used blocking agents are bovine serum albumin (BSA), casein (or a solution of non-fat dry milk), gelatin, or normal serum obtained from the species of animal in which the secondary antibodies are made. Avoid using serum of the same species as the primary antibodies. Typically, the protein solutions are used at concentrations of 1 to 10% in buffer and the samples are treated after permeabilization for 10 to 30 minutes. It is also advisable to include a small amount of detergent (if the sample is to be permeabilized) to compete for hydrophobic interactions with the antibodies. In this case, low (around 0.1%) concentrations of Triton X-100 should be used, for example 3% BSA in PBS with 0.1% Triton X-100 or 5% fetal calf serum (FBS) in PBS with 0.1% Triton X-100 for 30 minutes at room temperature works well.

Antibodies

Antibodies for immunofluorescence are divided into two categories: primary and secondary antibodies. These two groups are classified based on whether they bind to antigens or proteins directly or target another (primary) antibody that, in turn, is bound to an antigen or protein. Primary antibodies can be labeled or unlabeled. Primary antibodies that have been covalently linked with a fluorescent moiety will have specificity and bind directly to the target as well as the tag necessary for imaging. This is known as direct immunofluorescence (Figure 2). By coupling the primary antibody with a fluorophore, direct immunofluorescence is faster than the indirect version because time-consuming washing and incubation steps are omitted. Thus, direct immunofluorescence is easier to handle and therefore suitable for the rapid analysis of samples in standardized experiments, for example in clinical practice. Unlabeled primary antibodies on the other hand will bind to the target but require a secondary antibody that will specifically recognize the antibody. This situation is called indirect immunofluorescence.

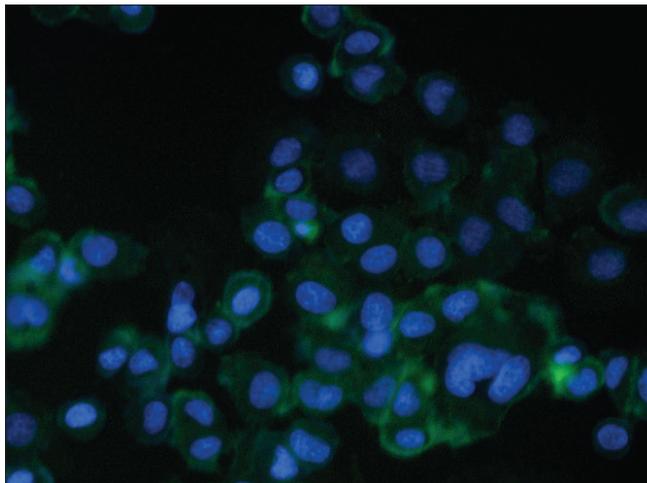


Figure 2. Directly labeled antibody fluorescence on unpermeabilized SK-BR-3 Cells. Anti-EGFR antibody covalently labeled with FITC and incubated with unpermeabilized SK-BR-3 cells counter stained with Hoechst 33342. Cells were imaged with a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek Instruments). Green fluorescence is located only on outer cell periphery as EGFR is a plasma membrane receptor.

Primary antibody binding to the specific antigen involves the Fab domain of the antibody, which in turn leaves the Fc domain exposed (Figure 3). The secondary antibody's Fab has been developed to recognize the Fc domain of the primary antibody. Since antibody's Fc domain is constant within the same animal class, only one type of secondary antibody is required to bind to many types of primary antibodies. This reduces the cost by labeling only one type of secondary antibody, rather than labeling various types of primary antibodies.

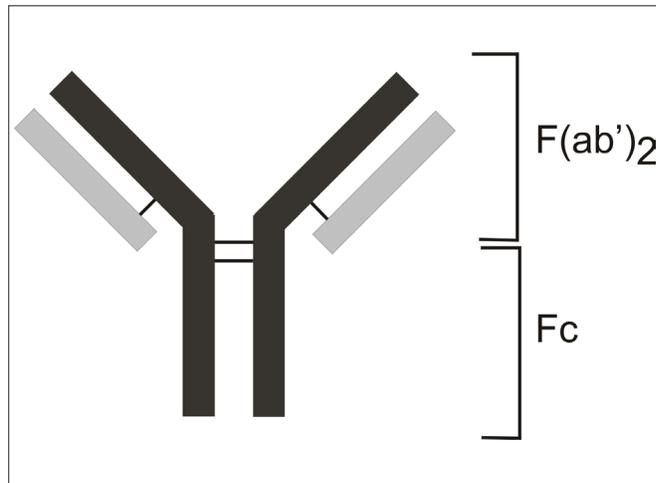


Figure 3. Structure of IgG Protein.

Primary Antibodies

Primary antibodies can be either polyclonal or monoclonal in nature. Polyclonal antibodies are a heterogeneous mixture of antibodies directed against various epitopes of the same antigen. As they are generated from different B-cell clones they are immunochemically dissimilar and can have different specificities and affinities. While a number of different animal species, including goat, swine, guinea pig and cow, are routinely used to produce polyclonal antibodies, rabbits are most frequently used. Monoclonal antibodies are a homogeneous population of immunoglobulin directed against a single epitope. These antibodies are generated from a single B-cell clone isolated from a single animal and as such have the same Fab structure. While the vast majority of monoclonal antibodies are produced from mice, increasingly, they are being produced from rabbits as well.

Binding affinity and the resultant titer towards its antigen is different for every antibody. The use of monoclonal antibodies removes much of the variability, but batch to batch concentrations can vary even with the same monoclonal due to aggregation or denaturation. As a result the antibody concentration required and the incubation time used can vary lot to lot. Optimal assay development requires that the primary antibody be titrated for best results. Too little antibody results in an artificially low signal, due to a lack of target saturation. Too much antibody can result in nonspecific binding despite have used a blocking step. Because of the reagent cost, it is paramount that only as much antibody as required be used. However, for a one-off experiment, or a small series of experiments the time, effort and expense required for a full antibody titration is often not warranted. Typically, a dilution of 1:1000 for the commercially available monoclonal antibodies is used. Because of the low amounts of protein in the sample, it is best to use a buffer containing BSA to dilute the antibody, for example, PBS 0.1% Triton X-100 supplemented with 30 mg/mL of BSA. This can be made in bulk, filter sterilized and aliquoted. Use only enough antibody dilution to cover the sample, - for a typical well in a 96-well microplate this is 20-25 μ L. Most of the commercially available antibodies have sufficient titer to bind high copy number targets in 30-60 minutes at room temperature. Results with low copy number targets often are improved by overnight incubation at 4 $^{\circ}$ C, and shaking can help as well. An incubation time of 60 minutes at room temperature works well for most experiments, but an overnight incubation can be used as a convenient end of the day stopping point for sample processing even with high target number epitopes.

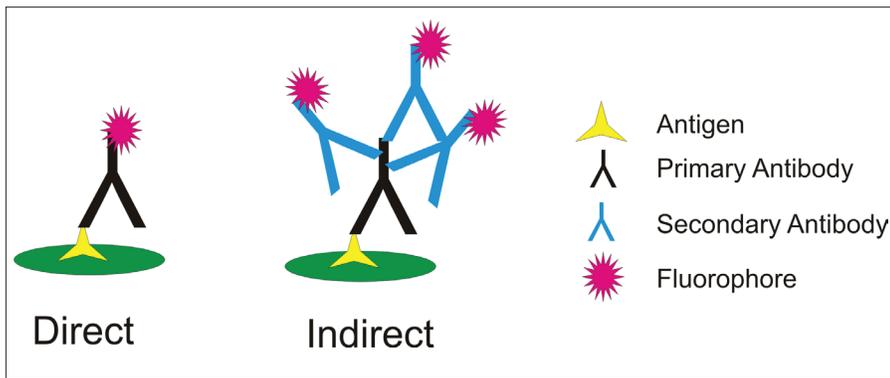


Figure 4. Direct vs. Indirect Antibody Labeling.

Secondary Antibodies

A secondary antibody is required with indirect immunofluorescence, where the primary antibody provides no means of detection and works by binding to a primary antibody, which directly binds to the target antigen. The use of secondary antibodies to indirectly detect target antigens requires additional process steps but can also offer significant advantages over labeled primary antibodies. Secondary antibodies increase the sensitivity through the signal amplification that occurs as multiple secondary antibodies bind to a single primary antibody (Figure 4). In addition, a given secondary antibody can be used with any primary antibody of the same isotype and target species, making it a more versatile reagent than individually labeled primary antibodies. This flexibility is a significant advantage of indirect immunofluorescence. Because the vast majority of primary antibodies are produced in just a few host animal species and most are of the IgG class, it is economical to produce and supply ready-to-use secondary antibodies for many methods and detection systems. From a relatively small number of secondary antibodies, many options are available for purity level, specificity and label type for a given application. A dilution of 1:500 for some commercially available secondary antibodies (Figure 5). As with primary antibodies, the low amounts of protein in the reagent makes it imperative that a buffer containing BSA or other non-specific protein be used to dilute the antibody, for example PBS 0.1% Triton X-100 supplemented with 30 mg/mL of BSA can be used for the dilution of secondary antibodies.

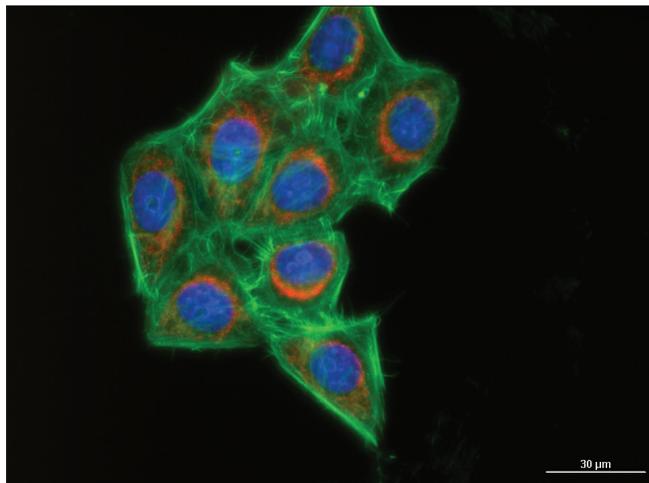


Figure 5. Fixed and three color fixed and stained HeLa cells. Mitochondria identified by a mouse anti-mitofilin primary antibody followed with a Rabbit anti-mouse IgG monoclonal antibody labeled with Texas Red (Red). Nuclei and actin filaments are identified by DAPI (blue) and AlexaFluor®-488-phalloidin (green) counterstaining respectively. Scale bar indicates 30 μm.

It is important that the secondary antibody employed in the experiment is raised against the host species used to generate the primary antibody. Polyclonal primary antibodies are usually raised in rabbit, goat, sheep or donkey and are generally IgG isotypes. The secondary antibody therefore, will typically be an anti-IgG antibody. Monoclonal primary antibodies are commonly raised in mouse, rabbit and rat. For example, if the primary monoclonal antibody is a mouse IgG, you will need an anti-mouse IgG.

The use of pre-absorbed secondary antibodies reduces the risk of cross reactivity with other antibodies and should be used with multi-color experiments when several primary antibodies and their corresponding secondary antibodies are used simultaneously.

Pre-adsorption is an additional processing step where the secondary antibodies are passed through a column matrix containing immobilized serum proteins from potentially cross reactive agents. For example, only antibodies specific to rabbit IgG will pass through a pre-absorbing column containing immobilized mouse, human and horse IgG whereas, antibodies cross reacting will bind and stay adsorbed to the matrix.

Washing

Between each process step, there is usually a wash step to remove unbound excess reagents. The buffer used for these steps should take into account the buffer used for the process steps. As described previously, it is best to use a buffer system that is isotonic to preserve cellular structure and close to physiological pH. Washing, as the name implies is a series of aspiration and buffer reagent additions performed in sequence. Depending on the volume used, 2-3 cycles is sufficient for washing, with the final step being an aspiration to remove the fluid prior to the addition of the next processing reagent. PBS is commonly used, and a surfactant such as 0.1% Triton X-100 or 0.05% Tween™ 20 can also be added. Note that it is important to make these steps in a timely fashion to prevent the sample from drying out.

Counter Stains

Counter stains are used for two different purposes; reduction of background fluorescence or the identification of cellular organelles. Blue dyes, such as Evans blue can be used to reduce non-specific background fluorescence with fluorescein labeled samples. Because these dyes can mask weakly fluorescent desired binding they are not recommended for routine use. More importantly is the use of counter stains to identify cellular organelles and provide information regarding signal localization.

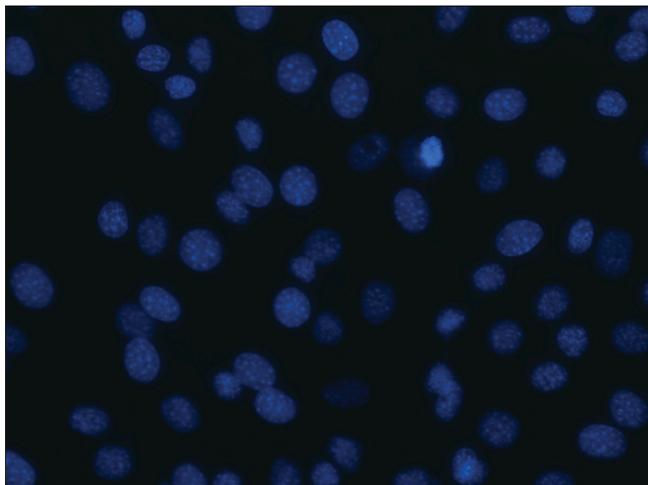


Figure 6. NIH 3T3 cell nuclei stained with DAPI.

The nucleus is the predominately targeted cellular organelle and can be identified through the use of stains that bind DNA (Figure 6). DAPI (diamidino-2-phenylindole) is a nuclear counter stain useful with multicolor fluorescent experiments. Its blue fluorescence differs significantly from fluorescein (green) or Texas red fluorescent probes used for other structures. It fluoresces brightly upon selectively binding to the minor groove of double stranded DNA. Its selectivity for DNA allows efficient staining of nuclei with little background from the cytoplasm. Other nuclear stains include Hoechst 33342, which is cell permanent and can be used with live as well as fixed cells, and propidium iodide, long used as a nuclear marker for flow cytometry which fluoresces in the red range. Far red dyes such as Draq5 can also be used a nuclear counter stain.

Fluorescently labeled phalloidin can be used as a counter stain for actin (Figure 7). This cyclic peptide isolated from the death cap mushroom (*Amanita phalloides*) has a very high affinity towards F-actin filaments. Because of its relatively small size and chemical stability, fluorescent derivatives are enormously useful in identifying actin filaments with high density labeling of the cytoskeleton which provides a means for the assessment of cellular morphology. As they are commercially available in a multitude of different colors they can be multiplexed with several other cellular probes.

Fluorescently tagged wheat germ agglutinin (WGA) can be used to stain for cell membranes. WGA is a carbohydrate-binding protein that selectively recognizes sialic acid and N-acetylglucosaminyl sugar residues which are predominantly found on plasma membrane proteins.

Unlike antibodies, counter stains usually do not have cross reactivity with one another and often can be combined into a single process step - the only limitation would be buffer compatibility. For example, DAPI (5-10 $\mu\text{g}/\text{mL}$) and phalloidin (20 nM) counter stains can be combined in a mixture of PBS 0.1% Triton X-100 from concentrated stock solution, freshly made on the day of the experiment. A 10-minute incubation at room temperature is sufficient to adequately stain fixed tissue culture cells.

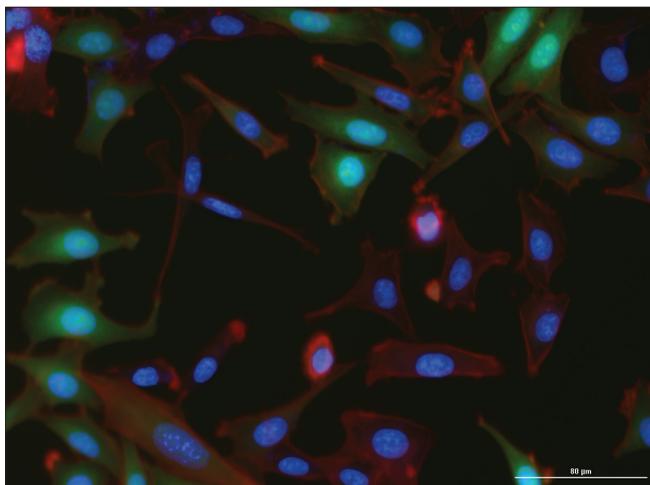


Figure 7. Fixed NIH 3T3 cells expressing GFP counterstained with DAPI and Texas Red labeled phalloidin. NIH3T3 cells cultured in 96-well plates were formaldehyde fixed then counter stained with DAPI and Texas red labeled phalloidin. Cells were imaged with a Cytation™ 3 using configured with DAPI, GFP and Texas Red light cubes.

Automation

Fixation and staining of slides or microplates is a multistep process with a number of reagent additions, wash steps and incubations (Figure 8). The processing of slides or coverslips is generally a low throughput operation and can usually be accomplished with a manual operation. Both fixtures can be treated using small dishes or in the case of slides dedicated Coplin jars can be used to store reagents or as vessels for wash media. Microplates, particularly 96- and 384-well plates with cells are much more amenable to automation. Because microplates have standardized well size, spacing and footprint, automation can be used to provide reagent addition and washing (aspiration and dispense). The small size of coverslips also allows them to be placed in the wells of low-density plates (6- and 12-well) for processing prior to adhering them to a slide.

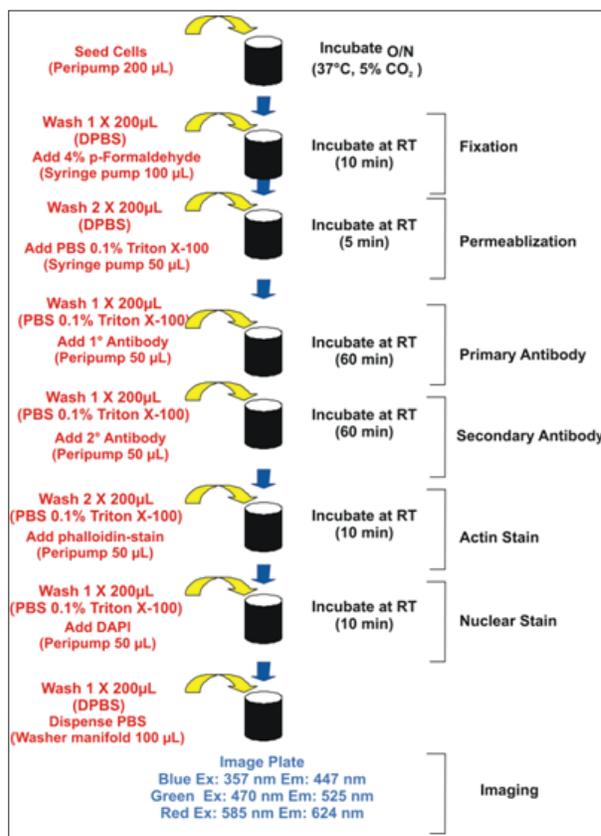


Figure 8. Automated Workflow for Cell Seeding, Fixation, Permeabilization and Three Color Staining Process. An EL406™ Combination Washer Dispenser (BioTek Instruments) was used to carry out the process steps for cell fixation, permeabilization and staining with three colors (DAPI, Alexa-Fluor® 488 phalloidin stain, and Texas red labeled secondary antibody) are indicated in red.

Storage

Storage of fixed and stained specimens can vary depending on the sample. Specimens on slides (or coverslips) can be treated with a mounting medium and sealed for long term storage. Mounting medium helps preserve the sample and raises the refractive index, which will improve performance with oil objectives. Mounting medium often has added agents to scavenge free radicals and reduce photobleaching. Prolong Gold (Life Technologies) and Fluoromount-G (SouthernBiotech) are examples of commercially available mounting media. It is important to rinse the slide/coverslip in distilled water prior to sealing as the salt from the PBS wash will precipitate out as crystals as it dries. After adhering the coverslip to the slide, allow the slide to dry overnight prior to sealing. Seal the edges of each coverslip with regular transparent nail polish and allow drying for 3 minutes; cells are now ready for imaging. Slides should be stored in a light tight container to prevent photobleaching. This will provide semi-permanent preparations that will last months if kept in the dark.

Microplates storage is more challenging. The small well size and well depth makes it almost impossible to seal the sample through the use of a coverslip. Microplates are better stored wet at 4 °C - PBS with 0.1% sodium azide can be used as a preservative. Plates are sealed with an adhesive plate seal and the plates wrapped in aluminum foil. While they will lose resolution over time, clear images can be obtained after one month in storage.

Final Comments

There is no “best” procedure for immunostaining and counter staining fixed cells on slides or microplates. There are numerous methods to fix, permeabilize, and stain cells, each with strengths and weaknesses. Immunostaining intracellularly involves detecting small molecules on interior cell structures often using much larger molecules, such as antibodies. Structures need to be stabilized, while at the same time holes need to be opened in membranes large enough to allow antibodies access to the interior. Different antibodies have different affinities and will often recognize different epitopes on the same protein, while different fixative methods will expose or mask different epitopes within the cell. While there is no one-size-fit-all technique for immunostaining, there are a number of different techniques that can be tested in order to optimize results.

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