In Situ Trypan Blue Staining of Monolayer Cell Cultures for Permanent Fixation and Mounting

Trypan Blue is one of the most commonly used dyes for assessing cell viability by dye exclusion assays. Dye exclusion assays are based on the principle that an intact cell membrane is necessary for the exclusion of certain dyes. Some other dyes used for this purpose include safranin, eosin, Congo Red, erythrocyan, nigrosin and Alcian Blue. Pappenheimer was the first to describe the use of Trypan Blue for analyzing cell viability. Since then, Trypan Blue viability staining has become one of the most common methods of determining loss of cell viability, particularly at the time of subculture, because the method is inexpensive, dependable and efficient. It is likely that other laboratories have also developed their own methodologies for applying Trypan Blue staining to adherent cells growing in monolayer culture, but this is the first report that actually describes a method allowing for fixation and permanent mounting of Trypan Blue-stained cell cultures grown on coverslips. Allison and Ridolpho described a method by which cells could be Trypan Blue-stained, fixed and permanently mounted on a slide, but they used cells grown in suspension and sedimented onto the slide rather than monolayer cultures grown on coverslips as reported here. If in situ Trypan Blue staining is done on monolayer cultures without fixation and permanent mounting, the water-soluble stain will eventually leach out of the cells and disappear. However, once fixed and permanently mounted, the Trypan Blue stain is permanently fixed into the tissue and will not fade over time. This method, because it is done in situ on monolayer cultures and incorporates a permanent mounting step, leads to several key advantages over traditional in situ Trypan Blue techniques. First, this method provides the convenience of analysis at any later time. Second, it greatly facilitates the use of computerized morphometric analysis to quantify the data. Finally, it allows for the use of double-staining techniques in conjunction with Trypan Blue, and we describe one such double-stain method subsequent to this report.

This method has been extremely useful in our laboratory’s cytotoxicity studies using both primary human neuronal cultures (that are heterogeneous in composition) and neuronal cell lines. The technique described here can be applied to any cell line, because Trypan Blue staining is known to be widely applicable to all cell types. The protocol is provided in Table 1.

Regarding step 4, note that many different Trypan Blue concentrations are seen in the literature, ranging from 0.04%–0.4%. The concentration can be varied within this range to achieve the desired staining intensity. Low concentrations around 0.04% are typically used in instances where the cells have been trypsinized, and it is likely that they will be exposed to the Trypan Blue for more than a few minutes, such as for cells in suspension at the time of subculture. Lower concentrations are necessary in these circumstances because the action of the trypsin alters the membrane of viable cells so that they will gradually take up Trypan Blue. However, because the membranes of cells in monolayer culture have not been altered by trypsinization, higher concentrations of Trypan Blue can be safely applied without compromising the integrity of viable membranes, provided that staining times are kept at 1 min or less. At a concentration of 0.2%, there is a slight variation in staining intensity among cell types, but this concentration works well for our primary neurons, glial cells and neuronal cell lines. Regarding step 5, other fixatives that we have tried that have no detrimental effects on the Trypan Blue staining include 2.5% glutaraldehyde for 30 min at 20°C–22°C and 4% PFA/1% glutaraldehyde for 10 min at 20°C–22°C or 20 min at 0°C. Post-fixes in 66% ethanol/33% glacial acetic acid for 5 min at -20°C also do not decrease the intensity of staining.

In summary, we have detailed a protocol for fixing and mounting coverslips of adherent cell cultures that have...
been permanently stained with Trypan Blue in situ. Although Trypan Blue staining may not be the most suitable dye exclusion assay for all in situ viability assays, it is still commonly used for monolayer cultures. For applications involving cytotoxicity, this method has been consistent and reproducible. Furthermore, the key innovation and strength of this protocol is that it is the only reported dye exclusion method we know that has been found suitable for permanent fixation and mounting in monolayer culture. Krause et al. (3) found that whereas Erythrosin B staining may be preferable to Trypan Blue staining for monolayer cells in culture, only the fluorescent form gave suitable staining results. The red form of Erythrosin B for light microscopy did not give effective staining, even at high concentrations. Therefore, because fluorescent staining is susceptible to photo-bleaching, such Erythrosin B staining would not be suitable for permanent fixation and mounting. Figure 1 shows a photograph of some Trypan Blue staining using this method in our primary cultures of human fetal neurons. Note the increase in Trypan Blue staining in the c-PAF (carbamyl-platelet activating factor: a phospholipid mediator known to cause both apoptosis and necrosis in human and rat neuronal cultures; Reference 2) treated cells (Figure 1b) compared to control (Figure 1a). Here it can be seen that the opportunities for morphological analysis of the nonviable (and viable) cells are clearly superior to those of more traditional methods of Trypan Blue staining. Because this application significantly expands the versatility of Trypan Blue viability assays and is the only published method of dye exclusion that allows for a permanent record of the staining in monolayer culture, we have found it to be extremely useful in our laboratory.

REFERENCES


Table 1. Trypan Blue Staining Method

1. Culture the cells on glass (or plastic) coverslips in 24- or 12-well cell culture plates according to the standard protocol. Treat with the desired conditions.

2. Using a centrifuge plate attachment, spin plates at 200×g to sediment any nonadherent cells (viable or dead) onto the coverslip. Remove all media.

3. If the culture medium contains serum, wash 1× for 3 min with phosphate-buffered saline (PBS) or serum-free culture medium to eliminate any residual serum (Trypan Blue has a strong affinity for serum proteins, which affects staining if serum is present; Reference 1). Centrifuge as above. Remove the wash.

4. Add a 0.2% Trypan Blue solution (0.4% Trypan Blue stock can be diluted 1:1 with PBS or serum-free culture medium) for 1 min.

5. Remove the Trypan Blue solution and immediately fix with 4% paraformaldehyde (PFA), pH 7.5, for 10 min at 20°–22°C, or 20 min at 0°C.

6. After fixation, rinse 3–4 times with PBS with gentle shaking until the PBS is clear of any residual blue color. Gently lift the coverslips to free Trypan Blue from underneath. Dehydrate the coverslips through sequential ethanol washes (1 min each in 70% and 95%, 3× for 1 min in 100%), followed by immersion in clearant (3× for 1 min in PRO-PAR Clearant [Anatech, Battle Creek, MI, USA] — a xylene substitute).

7. Finally, mount the coverslips onto glass slides with a compatible permanent mounting solution such as REFRAX Mounting Medium (Anatech).

Figure 1. Trypan Blue staining on primary cultures of human fetal neurons. Cultures are approximately 65% neuronal, 35% glial, taken from 2nd-trimester human fetal cerebral cortical tissue, in strict accordance with NIH and University of Rochester guidelines for human fetal tissue. Photographs show cells treated with either: (A) vehicle control or (B) 250 ng/mL c-PAF for 24 h, then stained and mounted by the method we describe here. Photographs were taken at 132× using an Olympus BX50 Light Microscope (Olympus, Lake Success, NY, USA) with ND25 and LBD filters and Nomarski optics (Olympus) to enhance contrast and add depth.


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Detection of Specific DNA-Binding Protein in HeLa Whole-Cell Extract

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DNA-binding proteins play a very important role in cellular processes such as replication, DNA recombination, transcription and gene regulation. The advent of molecular biology has greatly enhanced ability in the detection, isolation and characterization of these proteins. Many methods, including gel mobility shift assay (GMSA), UV cross-linking, DNase footprinting, methylation and uracil interference assay, have been successfully used in the study of DNA-binding proteins. Silva et al. (3) reported a method known as Southwestern hybridization, in which proteins are resolved on a sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto nitrocellulose. Transferred proteins are subjected to hybridization using a uniformly 32P-labeled DNA probe. The use of this method has greatly enhanced the detection sensitivity of DNA-protein interaction. However, the transfer of proteins onto a nitrocellulose membrane inevitably causes some protein loss, which can be detrimental to detection, especially when such protein has a low concentration. We report a simplified Southwestern analysis that produces comparable resolution but greatly shortens the experimental time and eliminates protein loss during protein transfer.

The adenovirus type 2 (Ad2) E1A upstream regulatory sequence (URS) contains an element that is regulated by 12-o-tetradecanoyl-phorbol-13-acetate (TPA), a phorbol ester (1). In an effort to identify TPA-inducible proteins that are able to bind to Ad2 E1A URS, we used Southwestern analysis. HeLa whole-cell extract was prepared as described by Manley et al. (2). Fifty micrograms of cell extract were resolved by 8% SDS polyacrylamide gel electrophoresis (PAGE). The gel was incubated in 200 mL renaturation buffer (50 mM NaCl, 10 mM Tris, pH 7.4, 2 mM EDTA, 0.1 mM dithiothreitol [DTT] and 4 M urea) with gentle agitation for 3 h at room temperature (or at 4°C for 18 h). Then the gel was incubated in 200 mL of blocking buffer H (10 mM Tris, pH 8.0, 2 mM MgCl2, 1 mM mercaptoethanol, 50 mM of NaCl and 5% Carnation® nonfat dry milk) for 2 h with gentle agitation at room temperature. A uniformly 32P-labeled polymerase chain reaction (PCR) fragment of Ad2 E1A URS (nucleotide [nt] -240 to -160) and 10 mg/mL of sonicated salmon sperm DNA fragments were added as nonspecific competitor to the blocking buffer and incubated for 3 h at room temperature. After hybridization, the gel was washed three times with buffer H for 10 min each. The gel was vacuum-dried and subjected to autoradiography for 16 h. The results (Figure 1) show that a 20-kDa protein (lane 2) binds specifically to Ad2 E1A URS at nt -240 to -160 in TPA-treated HeLa cells. While in control-cell extract (lane 1), a 45-kDa protein binds specifically to the same Ad2 E1A URS region. The same gel was stained with Coomassie® blue and showed that equal amounts of protein extract were loaded in each lane (data not shown).

Figure 1. In vitro DNA-protein hybridization using a uniformly PCR-labeled Ad2 E1A URS probe and HeLa whole-cell extract. Lane 1: dimethyl sulfoxide (DMSO)-treated cell extract; lane 2: TPA-treated cell extract.