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Protocol for adhesion and immunostaining of lymphocytes and other non-adherent cells in culture

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Immunostaining of non-adherent cells is commonly performed after adhesion of cells onto microscope slides either using cytocentrifugation or with the help of charged coating substrates. These techniques, however, require either specialized equipment or significant preparation time. Here, we describe a method for immunofluorescent staining of lymphocytes within multi-well culture plates, where cells suspended in phosphate buffered saline (PBS) are adhered to either the plastic well bottom or glass coverslips by gravity sedimentation. This technique requires only common laboratory materials, no coating steps, and allows for densely adherent cell coverage with 1 × 10^6 cells. Our data show that suspension of cells in PBS, but not serum-containing growth medium, allows for adhesion to plastic or glass after 30 min of gravity sedimentation. We show that this method is applicable for immunofluorescent staining of both primary human lymphocytes and immortalized lymphoma cells, and that it preserves cell morphology.

Immunocytochemistry is a common laboratory technique used to visualize the cellular expression of proteins through immunofluorescent staining and microscopy analysis. When analyzed by confocal microscopy, immunofluorescence is a powerful tool that can help determine the subcellular localization of proteins with high resolution, providing clues to protein function and interactions. The basic immunofluorescence staining procedure involves cell fixation, permeabilization, blocking, antibody binding, staining of nuclei, and sample mounting, with numerous washing steps in between.

While naturally adherent cells such as fibroblasts (1) and keratinocytes (2) can be grown directly on plastic cell culture plates or glass coverslips and are resistant to repeated washing, cells cultured in suspension, such as lymphocytes, must be purposely adhered during the immunostaining procedure. Commonly, this is performed by cytocentrifugation (3,4), in which a suspension of cells is pipetted into a cell-concentrating apparatus mounted to a microscope slide and subsequently centrifuged such that the cells are flattened against the glass. However, there are several drawbacks to cytocentrifugation, including equipment cost, inefficiency when preparing a large number of samples, and mildly distorted morphology that can attenuate cytoplasm and exaggerate the size of nucleoli (5–7). Another technique for adherence of suspension cells involves coating of plastic or glass surfaces with charged substrates such as poly-L-lysine to induce cellular attachment (8,9). However, this requires significant preparation time in the form of overnight treatment and experimental planning.

Here, we present a method that achieves results equivalent to the aforementioned techniques with benefits in terms of cost and time savings as well as increased efficiency in processing multiple samples (protocol included in the Supplementary Material). Our method is carried out within individual wells in a 12-well culture plate, allowing for streamlined processing of multiple samples and eliminating the need for handling separate microscope slides during the staining process. A No. 1.5 glass coverslip is placed onto bottom of each of the wells to be used, allowing for the adhered and stained cells on the coverslip to be transferred onto a microscope slide at the end of the procedure. It should be noted that this method can also be performed without the use of a coverslip, as cells can be attached directly onto the plastic bottom of the culture plate and analyzed with an inverted fluorescence microscope. This option may be suitable for experiments where rapid screening of protein expression is desired, and high-resolution confocal analysis is unnecessary (see Figure 1 for comparison).

METHOD SUMMARY

Cells suspended in PBS are adhered either to glass coverslips placed at the bottom of a multi-well culture plate or to the plastic well bottom itself, via gravity sedimentation. Adhered cells can be fixed and immunostained within the well of the culture plate and then analyzed using fluorescence or confocal microscopy.

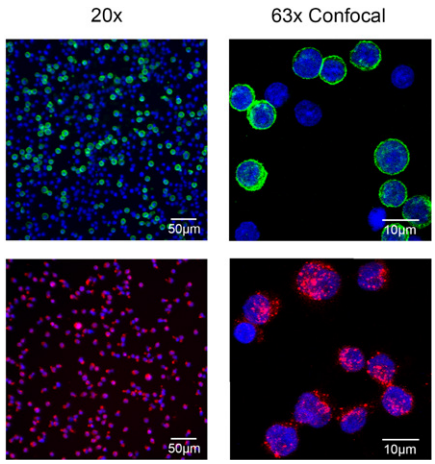

Figure 1. Immunostaining of lymphocytes analyzed by fluorescence and confocal microscopy. Immunofluorescent staining of CD4 (green, upper panel), a marker of helper T cells, showed membrane expression in a subset of primary human lymphocytes expanded from peripheral blood mononuclear cells. In PB2B cutaneous T-cell lymphoma cells, immunofluorescence of CTAGE1 (cutaneous T-cell lymphoma antigen 1; red, lower panel) demonstrated speckled cytoplasmic expression. Cell nuclei (blue) were stained using Hoechst dye. Images (20×) of cells adhered directly to the bottom of a well in a 12-well culture plate (left panels) were acquired using an inverted fluorescence microscope. Images (63×) of cells adhered to a coverslip placed at the bottom of a well and mounted to a microscope slide (right panels) were acquired using a confocal microscope. The protocol described here was followed using 1.0×10^6 cells per well.

Cells to be analyzed are pelleted by centrifugation at 250 × *g* for 5 min, and the growth medium supernatant is aspirated and replaced with PBS at a concentration of ~1×10^6 cells/mL. A P1000 pipette is used to gently mix and transfer 1 mL cell suspension into a culture plate well, with subsequent prodding of the well bottom with the pipette tip to ensure that the coverslip remains in place. The culture plate is then left stationary for 30 min at room temperature to allow for sedimentation and adhesion of cells onto the coverslip or well bottom. Although sedimentation of as few as 5×10^5 cells will result in relatively uniform adhesion, we have found that optimum cell density is achieved by using at least 1×10^6 cells (Figure 2A). Furthermore, we have found that suspension of lymphocytes in PBS, but not serum-containing growth medium, allows cells to adhere to plastic. Notably, cells suspended in serum-containing lymphocyte growth medium remain largely in suspension after 30 min of gravity sedimentation unless the adhesion surface is coated with a charged substrate (Figure 2B).

After cells have been adhered, the culture plate is slightly tilted, and the PBS suspension containing any remaining non-adherent cells is gently aspirated off. Adherent cells are then fixed for 10 min at room temperature with 500 µl 10% formalin pipetted along the side of the well. Fixed cells are washed once with 500 µl PBS for 5 min before being permeabilized with 500 µl 0.5% Triton X-100 for 10 min. Permeabilized cells are washed once again with PBS for 5 min and are then blocked with 500 µl 1% BSA in PBS for 30 min. The blocking solution is replaced with 250 µl of the primary antibody diluted in 1% BSA and left to incubate for 1 h at room temperature or overnight at 4°C. Cells are subsequently washed 3 times with PBS for 5 min and incubated with 250 µl of the secondary antibody diluted in 1% BSA for 1 h at room temperature, protected from light. Cells are again washed 3 times with PBS for 5 min. At this point, nuclei are counterstained with Hoescht dye at a working dilution for 10 min and then washed twice with PBS for 5 min to remove excess dye. A drop of anti-fade fluorescence mounting medium is added to the center of a microscope slide, and the coverslip is gently transferred cell-side down onto this surface after being gently lifted from the well bottom using sharp-tipped tweezers. After allowing the mounting medium to set for 15 min, the slide can be analyzed by fluorescence or confocal microscopy. If no coverslip was used, cells adhered to the well bottom and submerged in PBS can be analyzed by inverted fluorescence microscopy. Culture plates can then be wrapped in aluminum foil and stored at 4°C for several weeks.

In this study, we used previously described, patient-derived immortalized PB2B cutaneous T-cell lymphoma (CTCL) cells (10,11) and primary lymphocytes expanded from freshly isolated peripheral blood mononuclear cells of a healthy donor. The latter samples were obtained with informed consent in accordance with the Ottawa Hospital approved Institutional Review Board (IRB) protocol/Declaration of Helsinki. Although we found our method to be applicable to

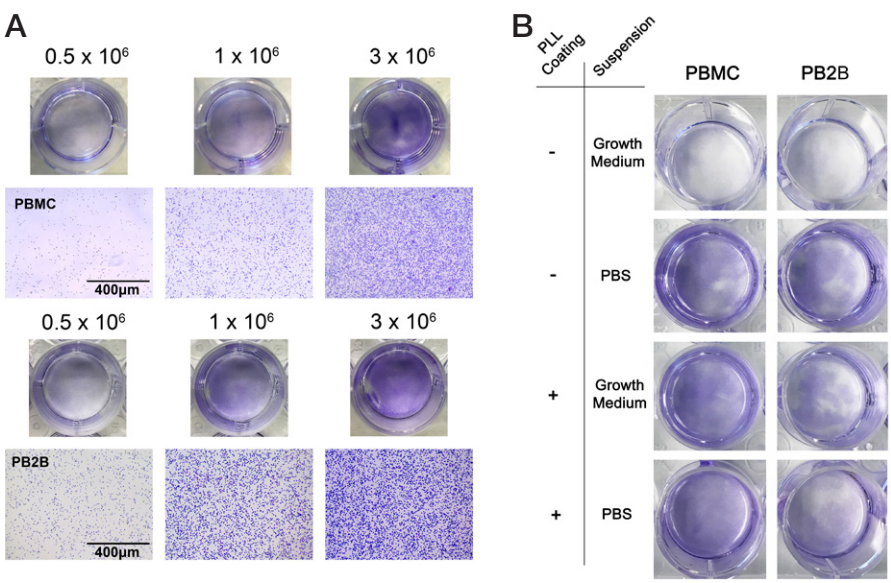

Figure 2. Lymphocytes suspended in PBS demonstrate uniform adhesion at 1×10^6 cells/mL after a 30-min gravity sedimentation without poly-L-lysine surface coating. (A) Crystal violet staining of lymphocytes expanded from human peripheral blood mononuclear cells (upper panel, PBMC) and immortalized PB2B cutaneous T-cell lymphoma cells (lower panel, PB2B) after 30-min gravity sedimentation of 0.5×10^6, 1×10^6, and 3×10^6 cells suspended in 1 mL PBS onto the wells of a 12-well culture plate. Cells were washed 10 times for 5 min with PBS before imaging. (B) Cells (1×10^6) were suspended in growth media (RPMI-1640 plus 10% fetal bovine serum) or PBS onto wells of a 12-well culture plate with or without overnight coating of wells with the attachment factor poly-L-lysine. Cells suspended in PBS, but not those suspended in growth media, were strongly adherent without poly-L-lysine coating. Cells were washed 10 times for 5 min with PBS before imaging.

both immortalized and primary lymphocytes, it is possible that the adhesion properties of other cell lines may differ. Nevertheless, we believe the protocol improves upon conventional methods in terms of cost and time savings, and that it will be useful for researchers studying lymphocytic diseases who seek an efficient method for non-adherent cell immunofluorescence staining using commonly available laboratory materials.

Author contributions

M.T. conceived, designed, and performed the experiments. M.T., J.G., and F.G. prepared the manuscript. I.L. supervised the study and corrected the manuscript.

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Competing interests

The authors declare no competing interests.

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