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

Matthew Tsang¹, Jennifer Gantchev², Feras M. Ghazawi¹, and Ivan V. Litvinov^{1,2}

¹*Division of Dermatology, University of Ottawa, Ottawa, Ontario, Canada* and ²*Division of Dermatology, McGill University, Montréal, Québec, Canada*

BioTechniques 63:230-233 (November 2017) doi 10.2144/000114610

Keywords: lymphocytes, non-adherent cells, protocol, immunocytochemistry, immunostaining

Supplementary material for this article is available at www.BioTechniques.com/article/114610.

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 immunofluores-

cence 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 (20x) 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 (63x) 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 $250 \times g$ for 5 min, and the growth medium supernatant is aspirated and replaced with PBS at a concentration of $\sim 1 \times 10^6$ 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 μ L 10% formalin pipetted along the side of the well. Fixed cells are washed once with 500 μ L PBS for 5 min before being permeabilized with 500 μ L 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 μ L 1% BSA in PBS for 30 min. The blocking solution is replaced with 250 μ L 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 μ L 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.

Acknowledgments

This work was supported by the new investigator funding program from the Ottawa Hospital Research Institute to I.L. and the Canadian Dermatology Foundation research grant to I.L., Joan Sealy Trust Cancer Research grant to I.L., and the Fonds de la recherche en santé du Québec (FRSQ) research grant to I.L. (# 34753 and 36769).

Competing interests

The authors declare no competing interests.

References

- Petrini, S., V. D'Oria, and F. Piemonte. 2015. Intracellular distribution of glutathionylated proteins in cultured dermal fibroblasts by immunofluorescence. *Methods Mol. Biol.* 1208:395-408.
- van Rossum, M.M., J. Schalkwijk, P.C. van de Kerkhof, and P.E. van Erp. 2002. Immunofluorescent surface labelling, flow sorting and culturing of putative epidermal stem cells derived from small skin punch biopsies. *J. Immunol. Methods* 267:109-117.
- Miotto, D., P. Boschetto, I. Bononi, G. Milani, C. Legorini, G. Cavallero, N. Lo Cascio, E. Zeni, et al. 2007. CC ligand 2 levels are increased in LPS-stimulated peripheral monocytes of patients with non-small cell lung cancer. *Respir. Med.* 101:1738-1743.
- Nowarski, R., O.I. Wilner, O. Cheshin, O.D. Shahar, E. Kenig, L. Baraz, E. Britan-Rosich, A. Nagler, et al. 2012. APOBEC3G enhances lymphoma cell radioresistance by promoting cytidine deaminase-dependent DNA repair. *Blood* 120:366-375.
- Peão, M.N., A.P. Aguas, C.M. de Sa, and N.R. Grande. 1992. Structural artifacts and advantages of cytocentrifugation of cells as viewed by scanning electron microscopy. *Scanning Microsc.* 6:281-285.
- Hanenberg, J. and S. Marionneaux. 2014. Cytospin preparation of AML in pleural fluid. *Blood* 123:3541.
- Stokes, B. 2004. Principles of Cytocentrifugation. *Lab. Med.* 35:434-437.
- Fischer, A.H., K.A. Jacobson, J. Rose, and R. Zeller. 2008. Preparation of slides and coverslips for microscopy. *CSH Protoc* 2008:prot4988.
- Mazia, D., G. Schatten, and W. Sale. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* 66:198-200.
- Litvinov, I.V., E. Netchiporouk, B. Cordeiro, H. Zargham, K. Pehr, M. Gilbert, Y. Zhou, L. Moreau, et al. 2014. Ectopic expression of embryonic stem cell and other developmental genes in cutaneous T-cell lymphoma. *Oncolmmunology* 3:e970025.
- Netchiporouk E., G.J., Tsang M., Thibault P., Watters A.K., Hughes J.D.M., Ghazawi F.M., Woetmann A., Ødum N., Sasseville D., Litvinov I.V. 2017. Analysis of CTCL cell lines reveals important differences between mycosis fungoides/sézary syndrome vs. HTLV-1+ leukemic cell lines. *Oncotarget*. (In press.)

Received 19 September 2017; accepted 17 October 2017.

Address correspondence to Ivan V. Litvinov, Room E02.6236, 1001 Decarie Blvd, Montréal, Québec, H4A 3J1. E-mail: ivan.litvinov@mcgill.ca

To purchase reprints of this article, contact: biotechniques@fosterprinting.com

UNITED STATES POSTAL SERVICE® Statement of Ownership, Management, and Circulation (Requester Publications Only)

1. Publication Title BioTechniques	2. Publication Number 0 7 8 8 - 0 2 0 5	3. Filing Date November 2017
4. Issue Frequency Monthly	5. Number of Issues Published Annually 12	6. Annual Subscription Price (if any)
7. Complete Mailing Address of Known Office of Publication (Not printer) (Street, city, state, and ZIP+4®)		
Informa, 52 Vanderbilt Ave NY, NY 10017		
8. Complete Mailing Address of Headquarters or General Business Office of Publisher (Not printer)		
Informa, 119 Faringdon Rd. London EC1R 3DA		
9. Full Names and Complete Mailing Addresses of Publisher, Editor, and Managing Editor (Do not leave blank)		
Publisher (Name and complete mailing address) Cheryl Wall, Publisher, 52 Vanderbilt Ave NY, NY 10017		
Editor (Name and complete mailing address) Nathan Blow, EIC, 52 Vanderbilt Ave NY, NY 10017		
Managing Editor (Name and complete mailing address)		
10. Owner (Do not leave blank. If the publication is owned by a corporation, give the name and address of the corporation immediately followed by the names and addresses of all stockholders owning or holding 1 percent or more of the total amount of stock. If not owned by a corporation, give the names and addresses of the individual owners. If owned by a partnership or other unincorporated firm, give its name and address as well as those of each individual owner. If the publication is published by a nonprofit organization, give its name and address.)		
Full Name	Complete Mailing Address	
Informa		
52 Vanderbilt Ave		
New York, NY 10017		
11. Known Bondholders, Mortgagees, and Other Security Holders Owning or Holding 1 Percent or More of Total Amount of Bonds, Mortgages, or Other Securities. If none, check box. ☒ None		
Full Name	Complete Mailing Address	
12. Tax Status (For completion by nonprofit organizations authorized to mail at nonprofit rates) (Check one) The purpose, function, and nonprofit status of this organization and the exempt status for federal income tax purposes: ☐ Has Not Changed During Preceding 12 Months ☐ Has Changed During Preceding 12 Months (Publisher must submit explanation of change with this statement.)		

PS Form 3526-R, July 2014 (Page 1 of 4) (See instructions page 4) PSN: 7530-08-000-8855 PRIVACY NOTICE: See our privacy policy on www.usps.com

UNITED STATES POSTAL SERVICE® Statement of Ownership, Management, and Circulation (Requester Publications Only)

13. Publication Title BioTechniques	14. Issue Date for Circulation Data Below November 2017
15. Extent and Nature of Circulation	Average No. Copies Each Issue During Preceding 12 Months
a. Total Number of Copies (Net press run)	73,755
(1) Outside County Paid/Requested Mail Subscriptions stated on PS Form 3541. (Include direct written request from recipient, telemarketing, and Internet requests from recipient, paid subscriptions including nominal rate subscriptions, employer requests, advertiser's proof copies, and exchange copies.)	51,285
b. Legitimate Paid and/or Requested Distribution (By mail and outside the mail)	17,598
(2) In-County Paid/Requested Mail Subscriptions stated on PS Form 3541. (Include direct written request from recipient, telemarketing, and Internet requests from recipient, paid subscriptions including nominal rate subscriptions, employer requests, advertiser's proof copies, and exchange copies.)	
(3) Sales Through Dealers and Carriers, Street Vendors, Counter Sales, and Other Paid or Requested Distribution Outside USPS®	
(4) Requested Copies Distributed by Other Mail Classes Through the USPS (e.g., First-Class Mail®)	
c. Total Paid and/or Requested Circulation (Sum of 15b (1), (2), (3), and (4))	68,883
d. Non-requested Distribution (By mail and outside the mail)	350
(1) Outside County Nonrequested Copies Stated on PS Form 3541 (include sample copies, requests over 3 years old, requests induced by a premium, bulk sales and requests including association requests, names obtained from business directories, lists, and other sources)	
(2) In-County Nonrequested Copies Stated on PS Form 3541 (include sample copies, requests over 3 years old, requests induced by a premium, bulk sales and requests including association requests, names obtained from business directories, lists, and other sources)	
(3) Nonrequested Copies Distributed Through the USPS by Other Classes of Mail (e.g., First-Class Mail, nonrequestor copies mailed in excess of 10% limit mailed at Standard Mail® or Package Services rates)	
(4) Nonrequested Copies Distributed Outside the Mail (include pickup stands, trade shows, showrooms, and other sources)	
e. Total Nonrequested Distribution (Sum of 15d (1), (2), (3) and (4))	350
f. Total Distribution (Sum of 15c and e)	69,233
g. Copies not Distributed (See Instructions to Publishers #4, (page #3))	4,522
h. Total (Sum of 15f and g)	73,755
i. Percent Paid and/or Requested Circulation (15c divided by 15f times 100)	100%

* If you are claiming electronic copies, go to line 16 on page 3. If you are not claiming electronic copies, skip to line 17 on page 3.

UNITED STATES POSTAL SERVICE® Statement of Ownership, Management, and Circulation (Requester Publications Only)

16. Electronic Copy Circulation	Average No. Copies Each Issue During Preceding 12 Months
a. Requested and Paid Electronic Copies	11,117
b. Total Requested and Paid Print Copies (Line 15c) + Requested/Paid Electronic Copies (Line 16a)	68,883
c. Total Requested Copy Distribution (Line 15f) + Requested/Paid Electronic Copies (Line 16a)	80,000
d. Percent Paid and/or Requested Circulation (Both Print & Electronic Copies) (16b divided by 16c x 100)	80,026
☒ I certify that 50% of all my distributed copies (electronic and print) are legitimate requests or paid copies.	
17. Publication of Statement of Ownership for a Requester Publication is required and will be printed in the November 2017 issue of this publication.	
18. Signature and Title of Editor, Publisher, Business Manager, or Owner	Date
Nora Pastenkos	11/3/17

I certify that all information furnished on this form is true and complete. I understand that anyone who furnishes false or misleading information on this form or who omits material or information requested on the form may be subject to criminal sanctions (including fines and imprisonment) and/or civil sanctions (including civil penalties).