

Chapter 11

Screening of Antigen-Specific Antibody-Secreting Cells

Hiroyuki Kishi, Aishun Jin, Tatsuhiko Ozawa, Kazuto Tajiri,
Tsutomu Obata, and Atsushi Muraguchi

Abstract

Screening of antigen-specific antibody-producing cells is a key step for obtaining antigen-specific monoclonal antibodies. In murine system, hybridoma between B-lymphocytes and myeloma cells is used to screen and produce antigen-specific monoclonal antibodies. In human system, good hybridoma-producing system is not available. Instead, transformation of B-lymphocytes with Epstein–Barr viruses is used to obtain antibody-secreting cell lines. Furthermore, phage-display system using molecular biology is recently used to obtain antigen-specific human monoclonal antibodies. Here, we describe the new method for screening antigen-specific antibody-secreting cells at single-cell levels using microwell-array chips. The system can be applied to screen antigen-specific antibody-secreting cells from any animal species.

Key words: Antigen-specific antibody, Antibody-secreting cell, Microwell-array chip, Immunospot array assay on chip

1. Introduction

Producing antibody-secreting cell lines from B-lymphocytes is the basal technique to screen and obtain antigen-specific antibodies. In murine system, hybridoma between B-lymphocytes and myeloma cells is produced to obtain antibody-secreting cell lines (1). In other animal species including human, efficient and stable hybridoma-producing system is not available. In human system, Epstein–Barr virus-transformed B-cell lines are often used to produce cell lines that produce human monoclonal antibodies (2, 3). In addition, library of phages that express antibodies on the surface is produced using recombinant molecular biology techniques and used to screen antigen-specific human antibodies (4, 5). Time-consuming and laborious step in both methods is that of preparing libraries of cells or phages that produce antibodies. It takes a month to several months to obtain the libraries and it is not so easy for any laboratories to

prepare good libraries (containing cells or phages producing antibodies with large varieties of antigen specificities), which makes it difficult to produce personal libraries from individual volunteers or patients who produce antigen-specific antibodies in blood.

Recently, we have developed microwell array chips that have 45,000–230,000 of microwells whose size and shape are just fit to capture single cells in each microwell (6–8). By arraying live human B-lymphocytes on the chip, we stimulated them with antigen and analyzed the alteration of intracellular Ca^{2+} concentration in antigen-stimulated B-lymphocytes on the chip. Since intracellular Ca^{2+} concentration transiently increases by the signals from B-cell antigen receptor, i.e., the cell surface immunoglobulin on B-lymphocytes, we can identify B-lymphocytes that produce antigen-specific antibodies on the chip. We have also tried and detected antigen-specific B-lymphocytes on the chip by arraying live human B-lymphocytes on the chip and detecting the binding of fluorescence-labeled antigen to B-cell antigen receptors on antigen-specific B-lymphocytes on the chip (9). We could successfully detect antigen-specific human B-lymphocytes on the chip using these protocols. The precision of antigen-specific B-lymphocyte detection, however, was not so high for these two protocols because of the background noises in the assay. So we have recently developed the third protocol to detect lymphocytes that produce antigen-specific antibodies on the chip (10). To this end, we analyzed antigen specificity of the secreted antibodies from single lymphocytes that were accommodated in microwells on the chip. As the corresponding microwell array chips, Love et al. have developed microengraving method that uses microwell array chips with microwells of 50 μm diameter and depth (11). For analyzing single cells, they need to adjust the cell concentration by limiting dilution. So it is difficult to trap single cells in most of wells. Deutsch et al. (12) as well as Biran and Walt (13) reported the microwell arrays that could accommodate single cell in each well, whereas their microwells were not suitable to detect antibody secretion from the accommodated cells and to retrieve the detected target cells. In these contexts, our microwell array chips are unique for their capacities of capturing single cells in each microwell, detecting antibody secretion from single cells, and enabling the retrieval of objective cells.

2. Materials

2.1. Preparation of Antigen-Coated Microwell Array Chips

1. Kimwipe™ paper (Kimberly-Clark Worldwide, Inc.).
2. Lipidure: 5% Lipidure BL-103 (NOF, Tokyo, Japan). Dilute the Lipidure to 0.01% (500-fold) in phosphate-buffered saline (PBS) before use. Keep it at room temperature until use.

2.2. Preparation of Antibody-Secreting Cells

3. Vacuum pump.
1. Heparin: Store at 4°C.
2. Syringe.
3. Needle: 21 G.
4. Ficoll-Conray solution: Lymphosepal I (IBL, Fujioka, Japan).
5. PBS: Weigh 8.0 g NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.24 g KH₂PO₄ and transfer to a 1-L glass beaker containing about 900 mL of water (ultrapure water prepared by purifying deionized and distilled water to attain a sensitivity of 18 MΩ cm at 25°C). Mix and make up to 1 L with water. Sterilize by autoclaving at 121°C for 20 min.
6. Hemocytometer.
7. Turk solution.
8. CD138 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (see Note 1).
9. AutoMACS pro (Miltenyi Biotec).
10. AutoMACS column (Miltenyi Biotec).
11. Cell culture medium: RPMI1640 (Invitrogen, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol (see Note 2).

2.3. Immunospot Array Assay on a Chip

1. Microwell array chip containing 90,000 microwells (10 µm in diameter) (see Note 3).
2. PBS.
3. Antigen (see Note 4).
4. Lipidure BL-103 (provided as 5 wt% solution from NOF Corporation, Tokyo, Japan) (see Note 5).
5. PBS-L: PBS containing 0.01% Lipidure BL-103.
6. Cy3-conjugated anti-IgG; store according to the manufacturer's instruction (see Note 6).
7. Cell Trace Oregon green 488 (Invitrogen): Dissolve in dimethyl sulfoxide at 1 mg/mL and store at -20°C. Dilute to 0.5 µg/mL just prior to use with PBS-L.
8. CO₂ incubator: 5% CO₂ in the air, 37°C.
9. Incubation box (see Note 7).
10. Fluorescence microscope with halogen lamp, optical filters to observe FITC (for observing Oregon Green) and Cy3.
11. Digital camera for a microscope, which can observe Oregon Green and Cy3.

2.4. Retrieval of Antigen-Specific Antibody-Secreting Cells

1. Fluorescence microscope.
2. Micromanipulator (TransferMan NK2, Eppendorf, Hamburg, Germany).
3. Microinjector (CellTram vario, Eppendorf).
4. Capillary (12 μm in diameter, Primetech, Tokyo, Japan).
5. Paraffin oil.

3. Methods

3.1. Preparation of Antigen-Coated Microwell Array Chips

Antigen is coated onto the chip overnight, and then the chip is used for immunospot array assay on a chip (ISAAC).

1. Add 100 μL of antigen (see Notes 4 and 8) in PBS onto the well area on a microwell array chip.
2. Put the chip in a humidified black box.
3. Incubate the chip overnight at 4°C or room temperature (see Note 9).
4. Wash the chip by aspirating the antigen solution with a Kimwipe paper (or a filter paper) and then adding 100 μL of PBS-L.
5. Repeat step 3.
6. Remove the air in microwells using a vacuum pump.
7. Incubate the chip for 15 min at room temperature.
8. Wash the chip as in step 3 using cell culture medium instead of PBS-L (see Note 10).
9. Use the chip for ISAAC.

3.2. Preparation of Antibody-Secreting Cells

Human experiments should be performed with the approval of the Ethical Committee at the belonging organization, and informed consent should be obtained from the subjects.

1. Collect 50 mL of peripheral blood into syringe from vaccinated volunteer at 7th day post vaccination (see Note 11).
2. Divide 5 mL of blood into 15-mL conical tubes that contain 5 mL of PBS.
3. Insert a sterile Pasteur pipette into blood (Fig. 1 and see Note 12).
4. Add 3 mL of Lymphosepal I to the tube through the Pasteur pipette. Lymphosepal I goes under the blood.
5. Centrifuge the tube at $740 \times g$ (2,000 rpm) for 30 min at room temperature (see Note 13).

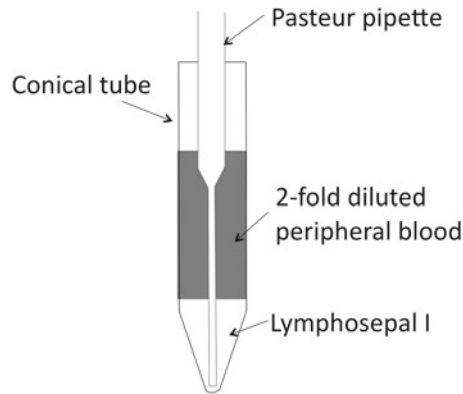


Fig. 1. Layering peripheral blood over Lymphosepal I. Sterile Pasteur pipette is inserted into twofold diluted peripheral blood. Lymphosepal I is, then, poured into the blood from top of the Pasteur pipette.

6. Mononuclear cells are layered over Lymphosepal I. Harvest the mononuclear cells using a Pasteur pipette (see Note 14) and transfer them to another 15-mL conical tube.
7. Dilute cells with PBS to 10 mL and centrifuge at $740\times g$ at room temperature.
8. Discard the supernatant with decantation, resuspend cells with approximately 10 mL of PBS, and centrifuge cells at $420\times g$ (1,500 rpm) for 5 min at room temperature.
9. Repeat step 9.
10. Discard the supernatant with decantation, and resuspend cells with 10 mL of PBS.
11. Count the cell number using Turk solution (see Note 15).
12. Centrifuge cells at $420\times g$ for 5 min at room temperature.
13. Remove the supernatant completely and add 90 μL of PBS per 10^7 cells.
14. Add 10 μL of CD138-microbeads to the cells.
15. Incubate the cells at 4°C for 15 min.
16. Add 1–2 mL of PBS to the cells and centrifuge them at $420\times g$ for 5 min at room temperature.
17. Remove the supernatant as completely as possible, and add 500 μL of PBS.
18. Separate CD138⁺ cells using AutoMACS Pro according to the manufacturer's instruction.
19. Count the cell number (see Note 16).

20. Spin down cells at $420\times g$ for 5 min at room temperature. Discard the supernatant and resuspend cells in cell culture medium to the cell density of 1.8×10^6 cells/mL.
21. Keep cells at room temperature until use.

3.3. Immunospot Array Assay on a Chip
(See Note 17)

When the chip is incubated, put the chip in a humidified black box in order not to dry the chip.

1. Remove the medium by aspirating it with a Kimwipe paper (or a filter paper), and then add 100 μ L of cells (1.8×10^5 cells).
2. Incubate the cells at room temperature for 5 min until the cells settle down into the wells.
3. Mix the cell suspension with a pipette for making cells outside of wells floating and incubate the chip for 5 min at room temperature (see Note 18).
4. Repeat step 3 once more.
5. Mix the cell suspension with a pipette for making cells outside of wells floating and remove the cell suspension by aspirating it with a Kimwipe paper (or a filter paper). Add 100 μ L of cell culture medium.
6. Repeat step 5 twice more.
7. Incubate cells at 37°C for 3 h in a humidified CO₂ incubator.
8. Remove the medium by aspirating it with a Kimwipe paper (or a filter paper), and then add 100 μ L of PBS.
9. Repeat step 8 twice more.
10. Remove PBS by aspirating it with a Kimwipe paper (or a filter paper), then add 100 μ L of Cy3-conjugated anti-IgG (1 μ g/mL in PBS-L), and incubate the chip at room temperature for 30 min.
11. Remove antibody solution with a Kimwipe paper (or a filter paper), and then add 100 μ L of PBS.
12. Repeat step 11 twice more.
13. Remove PBS by aspirating it with a Kimwipe paper (or a filter paper), then add 100 μ L of Cell Trace Oregon green 488 (0.5 μ g/mL in PBS-L), and incubate at room temperature for 5 min (see Note 19).
14. Wash the chip as in step 11 three times.
15. Observe antibody secretion with Cy3 signals (see Note 20) and cells with Oregon green signals under a fluorescence microscope (see Fig. 2).

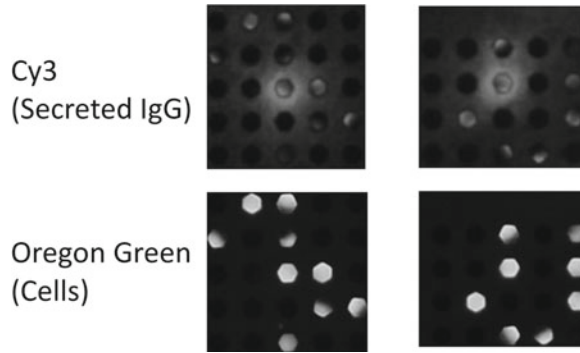


Fig. 2. ISAAC signals. *Top*, Cy3 signals showing secreted IgG. *Bottom*, Oregon green signals showing cells in microwells.

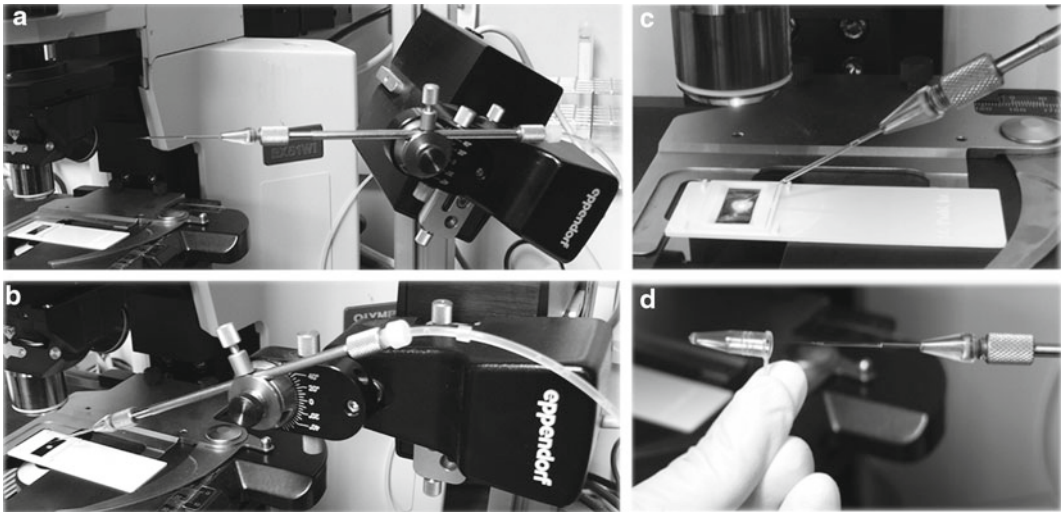


Fig. 3. Cell retrieval with micromanipulator. (a) Position of capillary and micromanipulator for cell ejection (ejection position). (b) Position of capillary and micromanipulator for cell retrieval (retrieval position). (c) Position of capillary for cell retrieval. (d) Transfer of a cell into a PCR tube.

3.4. Retrieval of Antigen-Specific Antibody-Secreting Cells

1. Observe the target cells that secrete antigen-specific IgG with a Cy3 circular signal under a fluorescence microscope (capillary and micromanipulator is at “ejection position” as in Fig. 3a) (see Note 21).
2. Bring the capillary and the micromanipulator to “retrieval position,” as in Fig. 3b. Adjust the tip of the capillary to the target-cell position under a microscope using a CellTransferman NK2 (Fig. 3c). Aspirate cells with a microaspirator (CellTram Vario).

3. Bring a capillary to the “ejection position,” and eject cells from the capillary by forming a tiny drop ($\sim 0.2 \mu\text{L}$) of buffer.
4. Transfer a drop containing a cell to RT-PCR buffer by adding the drop to the buffer (Fig. 3d, see Note 22).

4. Notes

1. For isolating human CD138-positive plasma cells.
2. 2-mercaptoethanol is optional.
3. The right of chip is wholly owned by Vivalis (Nantes, France).
4. Antigen should be dissolved in PBS in the absence of stabilizer, such as bovine serum albumin. It is recommended to check whether good and specific signals are obtained in enzyme-linked immunosorbent assay (ELISA) by using the same antigen- and positive-control antiserum.
5. Blocking reagent: Any other blocking reagents can be used, if the blocking reagents give good signal/noise ratio in ELISA. We use Lipidure because it shows blocking effect very rapidly and efficiently.
6. To detect antigen-specific IgG-secreting cells, use anti-IgG (Fc specific). When you use anti-IgG (whole molecule), the reagent contains anti-immunoglobulin light-chain antibodies and it reacts with other class of antibody, including IgM, IgA, and IgE.
7. For the incubation of chip, the chip was put in a humidified dark box as shown in Fig. 4.
8. The optimum concentration should be determined for each antigen. Usually, $1\text{--}10 \mu\text{g/mL}$ antigen is enough.
9. When the antigen is labile, coat the chip with antigen at 4°C . When the antigen is stable, coat the chip with the antigen at room temperature.

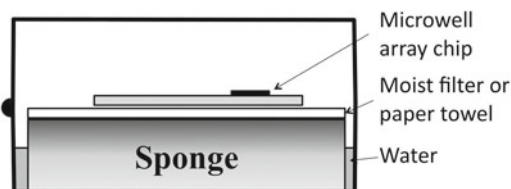


Fig. 4. Humidified dark box for the incubation of the microwell array chip. To avoid drying out of the solution on the chip during the incubation, microwell array chip was incubated in the humidified box. Sponge was humidified with water and a wet paper towel or filter paper was put on it. The chip was put on the filter paper for the incubation.

10. Be careful not to dry the chip. When aspirating the buffer, add the medium as quickly as possible.
11. The number of antigen-specific antibody-secreting cells that circulate in peripheral blood peaks 7 days after the vaccination, and then rapidly decreases within several days (14).
12. Glass Pasteur pipettes are sterilized at 180°C for 2 h in a dry incubator.
13. Brake should be off.
14. You can first remove the upper layer that contains plasma and most of platelets and then harvest the mononuclear cells.
15. Mix cells well with a Pasteur pipette. Harvest 10 μL of cell suspension and mix with 90 μL of Turk solution. Mix cell suspension well, transfer an aliquot of cell suspension to a hemocytometer, and count the cell number.
16. Volume of CD138-positive cells is approximately 2 mL after separation by AutoMACS Pro.
17. The ISAAC is covered by patents that have been exclusively licensed to Vivalis (Nantes, France).
18. The cells inside of wells tend to stay in the wells during the mixing of cell suspension. Mix cells mildly but strongly enough to float cells outside of wells.
19. Oregon Green fluorescence is strong and can be detected with optical filter for Cy3. Too strong Oregon Green fluorescence interfered for the observation of Cy3 signals. Adjust the concentration of Oregon Green and incubation time to label cells with optimal strength of the fluorescence signals.
20. Antibodies secreted from a cell that is in a microwell are diffused out to all directions. The bound secreted antibody on the chip is observed as a circular signal of Cy3. It looks like a doughnut and is easily distinguished from noise signals.
21. Be careful not to puncture fingers or eyes with the glass capillary. Wear glasses to protect eyes.
22. Be careful not to puncture fingers or eyes with a capillary. Wear glasses to protect eyes.

Acknowledgment

This work was supported by grants from the Toyama Medical Bio-Cluster Project and Hokuriku Innovation Cluster for Health Science of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497.
2. Kozbor, D., and Roder, J. C. (1981). Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein-Barr virus technique. *J Immunol* 127, 1275–1280.
3. Traggiai, E., Becker, S., Subbarao, K., Kolesnikova, L., Uematsu, Y., Gismondo, M. R., Murphy, B. R., Rappuoli, R., and Lanzavecchia, A. (2004). An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 10, 871–875.
4. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552–554.
5. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annu Rev Immunol* 12, 433–455.
6. Yamamura, S., Kishi, H., Tokimitsu, Y., Kondo, S., Honda, R., Rao, S. R., Omori, M., Tamiya, E., and Muraguchi, A. (2005). Single-cell microarray for analyzing cellular response. *Anal Chem* 77, 8050–8056.
7. Tokimitsu, Y., Kishi, H., Kondo, S., Honda, R., Tajiri, K., Motoki, K., Ozawa, T., Kadowaki, S., Obata, T., Fujiki, S., Tateno, C., Takaishi, H., Chayama, K., Yoshizato, K., Tamiya, E., Sugiyama, T., and Muraguchi, A. (2007). Single lymphocyte analysis with a microwell array chip. *Cytometry A* 71, 1003–1010.
8. Ozawa, T., Kinoshita, K., Kadowaki, S., Tajiri, K., Kondo, S., Honda, R., Ikemoto, M., Piao, L., Morisato, A., Fukurotani, K., Kishi, H., and Muraguchi, A. (2009). MAC-CCD system: a novel lymphocyte microwell-array chip system equipped with CCD scanner to generate human monoclonal antibodies against influenza virus. *Lab Chip* 9, 158–163.
9. Tajiri, K., Kishi, H., Tokimitsu, Y., Kondo, S., Ozawa, T., Kinoshita, K., Jin, A., Kadowaki, S., Sugiyama, T., and Muraguchi, A. (2007). Cell-microarray analysis of antigen-specific B-cells: single cell analysis of antigen receptor expression and specificity. *Cytometry A* 71, 961–967.
10. Jin, A., Ozawa, T., Tajiri, K., Obata, T., Kondo, S., Kinoshita, K., Kadowaki, S., Takahashi, K., Sugiyama, T., Kishi, H., and Muraguchi, A. (2009). A rapid and efficient single-cell manipulation method for screening antigen-specific antibody-secreting cells from human peripheral blood. *Nat Med* 15, 1088–1092.
11. Love, J. C., Ronan, J. L., Grotenbreg, G. M., van der Veen, A. G., and Ploegh, H. L. (2006). A microengraving method for rapid selection of single cells producing antigen-specific antibodies. *Nat Biotechnol* 24, 703–707.
12. Deutsch, M., Deutsch, A., Shirihai, O., Hurevich, I., Afrimzon, E., Shafran, Y., and Zurgil, N. (2006). A novel miniature cell retainer for correlative high-content analysis of individual untethered non-adherent cells. *Lab Chip* 6, 995–1000.
13. Biran, I., and Walt, D. R. (2002). Optical imaging fiber-based single live cell arrays: a high-density cell assay platform. *Anal Chem* 74, 3046–3054.
14. Wrammert, J., Smith, K., Miller, J., Langley, W. A., Kokko, K., Larsen, C., Zheng, N.-Y., Mays, I., Garman, L., Helms, C., James, J., Air, G. M., Capra, J. D., Ahmed, R., and Wilson, P. C. (2008). Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* 453, 667–671.