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Flow cytometry-based method for rapid and high-throughput screening of hybridoma cells secreting monoclonal antibody

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Monoclonal antibodies (mAbs) are a valuable biomaterial for basic life sciences and industrial purposes. The production of the mAb is time and effort intensive. In this report, we established a time- and labor-saving method for the mAb production. Because membrane-type immunoglobulin on a hybridoma cell surface and its secreted form, called as antibody, share the same binding property to the antigen, the fluorescence-labeled antigen bound to membrane-type immunoglobulin can be used as a screening marker. In the method, a hybridoma labeled by a fluorescent antigen was selected and sorted singly into 96-well plate using flow cytometer. Model experiments indicated that the method is highly efficient to obtain good mAbs suitable for Western blotting and immunofluorescence. Notably, most mAbs established by this method belonged to the IgG isotype, which is preferred over the IgM counterpart. Using a high-throughput flow cytometer, the method avoids tedious repeated screening and cloning processes. Because the method uses conventional myeloma for cell fusion and all reagents required in this method are commercially available, all research laboratories can apply the method to obtain mAbs efficiently.

[**Key words:** Monoclonal antibody; Flow cytometry; Hybridoma; Myeloma; Peptide antigen]

Hybridoma technology invented by Köhler and Milstein in 1975 (1) opened the door for mass production of monospecific antibodies, called monoclonal antibodies (mAbs). A mAb can bind to a specific molecule with high specificity and affinity and is considered a highly sensitive detection probe. Numerous mAbs have been developed, which have contributed to the advancement of basic life sciences in post-genomic era. MAbs also attract a lot of attention as biopharmaceutical products, and their market as an antibody medicine is expanding and is estimated to reach $215 billion in 2020 (2). Therefore, mAbs should be highly-functional biomaterials in basic biosciences and industries.

There are two prominent methods to generate mAbs, immunization-hybridoma technology and *in-vitro* surface display method. Although hybridoma technology is a widely used method, immune tolerance and epitope dominance may obstruct the generation of functional antibodies, such as neutralizing or functional antibodies (3). Surface display methods can overcome these intrinsic issues, however, it may be difficult to directly get a high-affinity antibody. Several methods to improve the binding affinity of the antibody *in-vitro* have been proposed. Activation-induced cytidine deaminase (Aicda) induces immunoglobulin class switch and somatic hypermutation in immunoglobulin heavy chain gene. In the surface display method, the exogenous expression of Aicda in antibody-expressing mammalian cells can improve the affinity of antibodies by affinity maturation (4,5). However, display methodologies are still a developing technology, and thus, the animal immunization-hybridoma technology has been used in many laboratories.

After cell fusions between B cells and myeloma cells, protocols of hybridoma technology include multistep screening and cloning processes which are labor-intensive and time-consuming. Therefore, the development of a simple and fast procedure for efficient establishment of hybridoma cell lines should reduce the burden on in-house production of desired mAbs.

Soluble antibodies (sIg) are secreted by B cells, and a single B cell displays about 200,000 to 500,000 membrane-type immunoglobulins (mIg) on their cell surface (6). The sIg and mIg of one B cell bind to same antigen with the same binding affinity, suggesting that the desired sIg-secreting cells can be selected using antigen–mIg binding as a selection marker.

Using a flow cytometer (FCM) and its sorting function, Parks et al. in the Herzenberg Laboratory reported an innovative method for separating and cloning the hybridoma cells using mIg as the selection marker (7). They used a fluorescent antigen to label the specific mIg. The FCM-based method is particularly helpful to save time and labor spent in tedious screening and cloning steps. They used fluorescent microsphere to increase the signal intensity. Their trial was provident, but the signal intensities were not strong

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enough to efficiently select the desired mAb-secreting cells. For expeditious hybridoma production, enhancement of the fluorescent signal on hybridoma is required. As exogenously expressing Iga increased mIg expression on hybridoma cell surfaces, Prices et al. (8) stably introduced the Iga gene into myeloma and used it as a fusion partner. The method, called Direct Selection of Hybridomas (DiSH), improved the cloning efficiency to establish a hybridoma cell line, but the engineered myeloma was legally protected. It should be very useful to establish a reliable and a nonproprietary FCM-based screening and cloning protocol.

Since the first report on FCM-based screening and cloning system by Parks et al. (7), there have been great advancements on the brightness of fluorophores and FCM sensitivity. Phycoerythrin (PE) is one of the brightest fluorophores and is used in many life science fields. The sensitivity of FCM has also been greatly improved. Considering these technical advancements, a reevaluation of the usefulness of the FCM-based screening and cloning method should be valuable.

We assessed the performance of this method using tubulin peptide antigen as a model system. After immunization and cell fusion, the hybridomas were screened and cloned by FCM-based screening and cloning system. Consequently, we developed a modern and simple FCM-based screening and cloning system which we called the MIHS (Membrane Immunoglobulin directed Hybridoma Screening and cloning) method. The method is reliable, highly efficient, and avoids any legal issue, thus, it should be useful in many laboratories. We also suggest that the method may preferentially select the structure-recognizing antibodies.

MATERIALS AND METHODS

Cell cultures The mouse myeloma cell line SP2/0-Ag14 (RCB0209) was provided by the Riken Bioresources Center (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT Japan. The cells were cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) with 10% heat-inactivated newborn calf serum (NBS: Biowest, Nuaillé, France) and recombinant mouse interleukin 6 (IL6: 1 ng/mL) purified from IL6-overexpressed *Escherichia coli* (9).

We maintained two hybridoma clones (3A1: IgG1 and 5H12: IgM) against mouse α-tubulin peptide (427–441 amino acid) generated using the conventional method in our laboratory and one clone (Red 8) established in this paper in RPMI 1640 with 10% NBS, 1 ng/mL recombinant IL6, and HT (0.1 mM hypoxanthine and 0.016 mM thymidine).

Expression of membrane-type immunoglobulin in hybridoma cells Expressions of mRNAs encoding mIg were examined in 3A1, Red8, and 5H12 hybridomas and splenocytes of BALB/cJcl female mouse. Total RNAs were extracted from the samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method (10) and were reverse-transcribed using oligo dT primer and PrimeScript reverse transcriptase (Takara Bio, Shiga, Japan) as per the manufacturer's instructions. The cDNAs were subjected to PCR analysis. The PCR cycles and primers used for detecting mIg and sIg for IgG and IgM isotypes are shown in Table S1. The expression of mIg proteins on hybridoma cell surface was examined by immunohistochemistry. These hybridomas were washed with PBS containing 0.1% BSA and were then incubated with 1:100 dilution of anti-CD16/32 mouse mAb (Tonbo Bioscience, Kobe, Japan) for 5 min at 4°C. Then, 2 μL of a 1:100 dilution of goat anti-mouse IgG (H + L)-PE antibody (Beckman Coulter, CA, USA) was added, and the cells were gently rotated for 60 min at 4°C. The cells were washed three times by PBS containing 0.5% BSA, stained by DAPI, and then used for microscopic observations by LSM 800 confocal microscope (Carl Zeiss, Jena, Germany) and FCM analysis.

Peptides, immunization, and cell fusion An antigen peptide of mouse tubulin which corresponds to mouse α tubulin amino acids 427–441 (NH_2-CALEKDYEEVGVDSVE-COOH) and the N-terminal-biotinylated tubulin peptide as screening peptides were synthesized (BEX, Tokyo, Japan).

The tubulin antigen peptide was conjugated with blue carrier protein (BCP: Pierce Biotechnology, IL, USA) according to the manufacturer's protocol and used for immunization. BALB/cAJcl female mice were immunized intraperitoneally with an emulsion of improved Freund's complete adjuvant and 100 μg peptide-BCP conjugate. Two weeks later, the mice were intraperitoneally boosted with 100-μg peptide-BCP conjugate in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4; pH 7.2). After three days of the final immunization, mice were sacrificed, and splenocytes were collected in PBS. The capsule of spleen was put in a nylon mesh bag filter, and cells were released by applying gentle pressure. Next, red blood cells were lysed in RBC buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, EDTA 0.1 mM). The cells were counted and fused with SP2/0-Ag14 cells at 3:1 ratio using freshly prepared 50% polyethylene glycol MW 4000 (Wako Pure Chemical Industries, Osaka, Japan) in 50-mM Hepes (pH 8.0). After extensively washing the fusion mixture with RPMI 1640, the cells were seeded in 10-mL RPMI 1640 supplemented with 10% NBS, recombinant IL6, and HAT (HT plus 0.4-μM aminopterin) in five 90-mm culture dishes and grown for six days.

The cultures after the fusion contained growing hybridoma and cell debris. The cell debris may disturb the cell separation by cell sorter. To remove the debris from the culture, we used density gradient centrifugation. All cultured cells were washed twice with PBS. The cells were resuspended in 20 mL of 13% OptiPrep density gradient medium (Alere Technologies, Oslo, Norway). RPMI 1640 medium (20 mL) was carefully layered on the cell suspension. After centrifugation at 800×*g* for 20 min, the white interphase was collected in 20-mL RPMI 1640 medium. The recovered cells were washed twice with PBS and adjusted to 5×10^5 cells/mL in RPMI 1640 medium supplemented with 10% NBS and IL6. Cell suspension (500 μL) was seeded into a single well of 24-well plate. The cells were cultured again overnight to recover the cell condition. The protocols of the animal experiments were reviewed and approved by the Ethics Committee on Animal Experiments of the Yokohama National University.

Antigen-mediated fluorescent labeling to hybridoma The purified fusion cells were subjected to the antigen-mediated fluorescent labeling. The N-biotinylated tubulin peptide (25 nmol) in the 500-μL medium was mixed in the well, and the plate was incubated for another 2 h. The cells were washed with PBS twice by centrifugation at 200×*g* for 3 min and were resuspended in 500-μL PBS with 2 μL of PE-streptavidin (Bio-Rad, CA, USA). After rotation for 1 h at room temperature, the cells were washed with PBS three times and were finally resuspended in 1 mL of RPMI 1640 supplemented with 2% NBS. The labeled cells were subjected to FCM analysis and confocal microscopic analysis. Before sorting, dead cells were stained by 7AAD.

Cell sorting The sorting was performed with a MoFlo Astrios instrument equipped with a 100-μm tip and three lasers tuned to 488 nm, 561 nm, and 633 nm. To avoid shear stress, the sheath pressure was adjusted to 20 psi. The basal fluorescence intensity was determined using the sample cells treated with PE-streptavidin alone. The cells carrying fluorescence intensities five times stronger than the basal fluorescence intensity were regarded as antigen-bound cells. The positive cells were sorted into a well of 96-well round bottom plate using the single-cell dispenser robot (CyClone software, Beckman Coulter, Inc., Indianapolice, IN, USA). These plates were incubated for 5–10 days at 37°C and 5% CO_2; then, we proceeded to the characterization of cloned hybridomas.

Characterization of mAbs obtained by the MIHS method For ELISA, antigen peptide (500 ng in PBS/well) was coated on a 96-well ELISA plate H (Sumitomo Bakelite, Tokyo, Japan) and incubated at 4°C overnight. The plate was washed once with PBS and then blocked with 5% nonfat skimmed milk in TBST (137 mM NaCl, 20 mM Tris–HCl, 0.1% Tween-20; pH 7.6) at room temperature for 2 h followed by washing three times with TBST. The plate was loaded with culture supernatants from sorted clones and was incubated for 1 h at room temperature. After three washes with TBST, the 1:10,000 diluted, peroxidase-conjugated goat anti-mouse IgG (H + L) secondary antibody (MBL, Nagoya Japan) was added to each well, and the plate was incubated for 1 h at room temperature. The plate was washed three times with PBS, and the bound antibody was visualized using TMB microwell peroxidase substrate system (Seracare, MA, USA). The absorbance values were read at 405 nm using PowerScan HT multiwell plate reader (DS-Pharma Biomedical, Osaka, Japan). For Western blotting analysis, the samples prepared from HEK293 cell (1×10^6 cells) were boiled, resolved by 12% SDS-PAGE, and transferred onto a polyvinylidene disulfide membrane (Immobilon-P SQ, Merck-Millipore, Darmstadt Germany). The membrane was divided into strips. The strips were blocked by 5% nonfat skim milk in TBST overnight, and were incubated with culture supernatants from the ELISA-positive clones for 1 h at room temperature. After three washes, 1:50,000 dilution of the peroxidase-conjugated goat anti-mouse IgG (H + L) secondary antibody was added to the strips and incubated for 1 h at room temperature. The strips were washed three times, and the signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore).

For immunocytochemistry, NIH 3T3 cells were plated on a slide glass equipped with FlexiPerm 8-well chamber (Greiner Bio-One, Kremsmunster, Austria). On the next day, the cells were rinsed two times with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were rinsed again with PBS and permeabilized in ice-cold methanol for 5 min. After washing the cells by PBS, they were blocked by 5% nonfat skim milk overnight. On the next day, the cells were incubated with 1:50 diluted culture supernatants from cloned hybridoma for 3 h, washed three times with PBS, and then incubated with 1:500 diluted Alexa 546-conjugated goat anti-mouse IgG (H + L) (ThermoFisher Scientific, MA, USA) in PBS for 45 min. After three washes by PBS, the cell nuclei were stained by DAPI (diluted 1:500) for 5 min, and the slide glass was covered with a cover slip and analyzed on the LSM 800 laser scanning microscope.

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