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Array-Based Analysis of Secreted Glycoproteins for Rapid Selection of a Single Cell Producing a Glycoprotein with Desired Glycosylation

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The therapeutic efficacy and in vivo biological function of a glycoprotein is significantly affected by its glycosylation profile. For the development of glycoproteins with therapeutic applications, selection of cell lines producing a glycoprotein with adequate glycoform is crucial. Here, we demonstrate an array-based analysis of secreted glycoproteins for rapid and efficient selection of a single cell producing a glycoprotein with desirable glycosylation. Our approach relies on microengraving and interrogation of glycoproteins produced by individual cells in a microwell array in terms of glycosylation profile as well as the produced amount. On the basis of statistical analysis of the interrogation, single cells which are predicted to produce a desired glycoprotein are selected, retrieved, and expanded. We applied the approach to human recombinant erythropoietin (rhEPO)-producing CHO cells and verified the selection of a single CHO cell that produces rhEPO with a high sialylation degree. Human erythropoietin (hEPO) bearing highly sialylated oligosaccharide was shown to display a much longer plasma half-life, resulting in high therapeutic efficacy. This method may find widespread use in the clonal selection for the production of other glycoproteins with specific glycosylation as well as analysis of the heterogeneity in cell populations in a high-throughput manner.

Glycoproteins constitute a major portion of protein-based drugs, accounting for about 70% of currently approved therapeutic proteins and drug candidates in development.[1,2] The glycan profile of glycoproteins, namely, the moiety and composition of oligosaccharide structures, varies with cell types, tissues, and species in which they are expressed. Consequently, a wide variety of glycosylation structures are generated, ranging from biantennary complex oligosaccharides to tetra-antennary fucosylated complex oligosaccharides. The glycosylation profile is known to greatly affect the function of a glycoprotein including its in vivo biological activity, immunogenicity, tissue targeting, and pharmacokinetics.[3−7] Typical glycoproteins whose functions are dependent on glycosylation include erythropoietin (EPO), antibodies, blood factors, interferons, and some hormones. In particular, the moiety or content of the terminal carbohydrate in the oligosaccharide has been shown to have a significant impact on the pharmacokinetics of therapeutic glycoproteins.[5]

In order to produce a glycoprotein with desired glycosylation, much effort has focused on both construction and selection of adequate cell lines and engineering of glycosylation pathways.[8,9] Several different types of host cells with varying abilities to perform glycosylation are available, and CHO cells are most widely used for the production of glycoproteins, because their glycosylation pattern is similar to that found in native human proteins.[1,6] Mammalian cell lines producing glycoproteins are usually constructed from a parental cell line by transfection of genes and repeated rounds of clonal selection with selective pressures. Even though the population of genetically engineered cell lines comes from a single parental cell, they are heterogeneous in glycosylation profile as well as growth rate and production yield.[6,7] Thus, the selection of cell lines having the capacity to produce glycoproteins with an adequate glycosylation pattern in high yield is crucial to the development of effective glycoproteins. The most common method for selection of individual cells includes repeated rounds of limiting dilution cloning (LDC) in conjunction with enzyme-linked immunosorbent assay (ELISA) in microtiter plates.[10] The LDC method is widely used due to its simplicity, but it is labor intensive and has low throughput. Many alternative approaches have been attempted such as gel microdrop technology, matrix-based secretion assay, microfluidic devices, cell-based microarrays,

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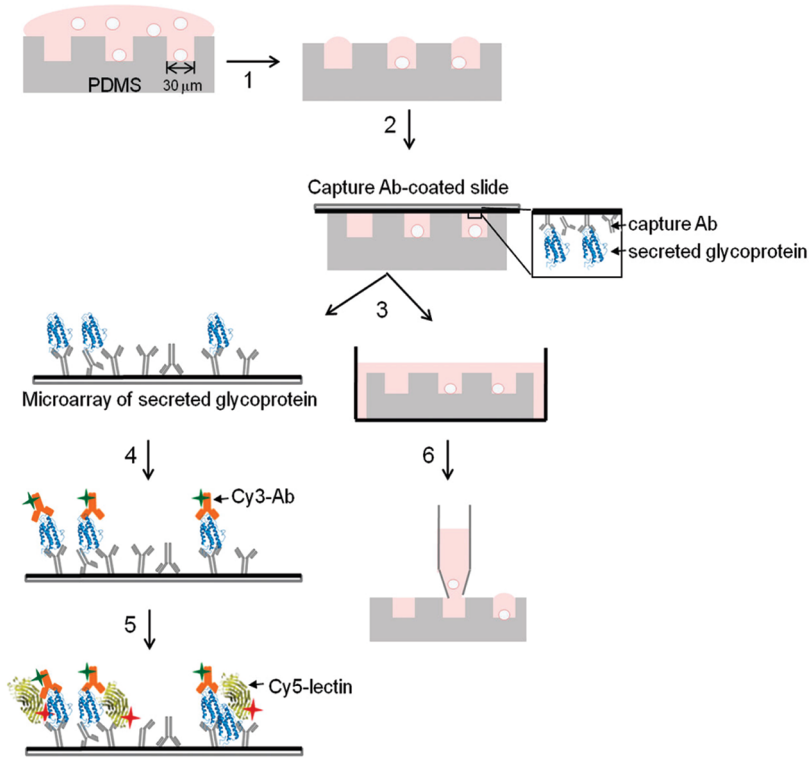

Figure 1. Schematic of an array-based analysis of glycoproteins secreted by individual cells in microwells for the selection of a single cell that produces a glycoprotein with desired glycosylation. (1) A solution of cells is deposited on a PDMS microwell array, and the cells are left to settle down into the microwells. (2) A glass slide coated with antiglycoprotein monoclonal antibody is placed over the microwell array, pressed, and incubated for 1 h to capture the glycoproteins secreted by individual cells in microwells. (3) After incubation, the cell-containing microwell array is separated from the glass slide and immersed in a reservoir of culture medium for retrieval after sorting. (4) The resulting slide is first treated with dye-labeled secondary antiglycoprotein antibody for probing the amount of secreted glycoprotein. (5) The slide is rinsed and further incubated with dye-labeled lectin which is specific for carbohydrate moiety of glycoprotein to probe the glycosylation profile. (6) On the basis of the statistical analysis of the fluorescent spots, corresponding single cells are selected, retrieved, and expanded for the production of the glycoprotein.

and micropallet arrays.[11−17] Recently, the microengraving method and immunospot array assay on a chip were developed for the selection of cells producing antigen-specific antibodies.[18−20] In the case of selecting cell lines producing a glycoprotein, detailed characterization of the produced glycoprotein is required at each round of clonal selection, which is still complicated and time-consuming despite many advances.[21−23]

Here, we demonstrate an array-based analysis of secreted glycoproteins for rapid selection of a single cell producing a glycoprotein with desired glycosylation using a glycan-specific lectin. Our approach relies on microengraving and dual probing of glycoproteins produced by individual cells in a microwell array to assess the glycosylation profile as well as the produced amount (Figure 1). On the basis of statistical analysis of the probing, single cells which are predicted to produce a desired glycoprotein are selected, retrieved, and expanded. As a proof of concept, we applied the method to the selection of a single CHO cell that produces recombinant human erythropoietin (rhEPO) with a high sialylation degree. Human erythropoietin (hEPO) bearing highly sialylated oligosaccharide was shown to display a much longer plasma half-life, resulting in high therapeutic efficacy.[5,7] This method enables the selection of a single cell producing a glycoprotein with specific glycosylation without additional characterization of its glycosylation profile in a high-throughput manner.

EXPERIMENTAL SECTION

Cells and Culture. The SCST3 cell line developed by introduction of three genes in a parent CHO cell was used as the starting rhEPO producer cell.[24] Cells were grown in MEMα media

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(Gibco) supplemented with 10% d-fetal bovine serum (d-FBS; Gibco), 3.5 g/L glucose, 1% antibiotic−antimycotic solution, 20 nM methotrexate (MTX), and 500 μg/mL zeocin (Invitrogen) at 37 °C (5% CO_2). The cells were subcultured every 3 days for long-term culture by trypsinization.

Dual Probing of rhEPO Isoforms. Selectively purified rhEPO having a pI value between 4.2 and 3.5 was provided by LG Life Science Inc. (Korea) and used as a standard. The standard rhEPO was digested for various reaction times (0, 2, 5, and 10 min) with sialidase−agarose from *Clostridium perfringens* (Sigma) and filtrated (YM30, Millipore) to remove digested sialic acids. The resulting rhEPO proteins were designated as isoform-1 to -4, respectively, according to the digestion times (0 min, isoform-1; 2 min, isoform-2; 5 min, isoform-3; 10 min, isoform-4). The pI value of digested rhEPO was determined using Novex IEF gels (Invitrogen) over a pH range of 3−7 according to the manufacturer's manual. Respective rhEPO isoforms with varying sialic acid content were serially diluted and spotted on the ultrathin nitrocellulose-coated glass slide (PATH slides, GenTel Bioscience, Madison) in nine spots with a 3 × 3 format using a robotic microarrayer (OMNIGRID, Genomics Solutions). After incubation for 1 h at 37 °C, the slide was treated with PBS supplemented with 1% BSA (bovine serum albumin) for 2 h at room temperature. For dual probing, rabbit anti-EPO polyclonal antibody (α-EPO) (Sigma) and *Maakia Amurensis* agglutinin (MAA) (Sigma) were labeled with Cy3 and Cy5 dyes (GE Healthcare), respectively, according to the manufacturer's manual. Following washing with Tris-buffered saline (TBS)/Tween 20 (0.05% w/v) (TBST) and deionized water, a solution of Cy3-conjugated rabbit anti-EPO polyclonal antibody (Cy3-α-EPO) (10 μg/mL in sodium phosphate (pH 7.0), 0.5% Tween 20) was loaded on the surface of the slide and incubated for 30 min at room temperature. The slide was rinsed with TBST and deionized water, and a solution of Cy5-conjugated MAA (Cy5-MAA; 100 μg/mL in sodium phosphate (pH 7.0), 0.5% Tween 20) was subsequently deposited on the surface of the slide and incubated for 1 h at room temperature. The resulting slide was thoroughly rinsed with TBST and deionized water, dried with nitrogen gas, and scanned with a GenePix 4100A scanner (Molecular Devices) using 532 and 635 nm lasers. The fluorescence intensities from Cy3 and Cy5 were measured and analyzed using GenePix Pro 6.0 software (Molecular Devices). To test steric hindrance, a single solution of Cy5-MAA was deposited on the spotted slide, and the fluorescence intensity of Cy5 was measured using a similar procedure as described above. The intensities taken from all spots were corrected for the background level.

Fabrication of Microwell Array. The microwell arrays were fabricated with poly (dimethyl-siloxane) (PDMS) (Sylgard 184, Dow Corning) using a photolithographic process. The arrays were composed of blocks of microwells with varying diameters: 25, 30, and 40 μm. The depth of the microwells and intermicrowell distance were fixed at 35 and 100 μm, respectively. A single set of arrays contained 45 × 45 microwells. A layer of photoresist (SU-8, Microchem) was patterned on a silicon wafer using a photomask to produce a master. Prior to molding, the patterned silicon surface was treated with (tridecafluoro-1,1,2,2,-tetrahydrooctyl) trichlorosilane (Gelest) using a vacuum evaporation method. The PDMS prepolymer was poured onto the master, followed by removal of gas in a vacuum chamber, and cured at 70 °C for 6 h in oven. After curing, the PDMS replica was peeled off and cut into small blocks containing a proper number of microwells with a razor blade. The array of PDMS microwells was bonded to a glass slide for easy handling by plasma treatment and used for further experiments.

Cell Deposition in a Microwell Array. The fabricated PDMS microwell array was sterilized by an autoclave under 1 kg/cm^2 for 15 min. To facilitate cell adhesion, the inside wall and the bottom of the microwells were coated with fibronectin by incubation with a fibronectin solution (50 μg/mL in PBS, Sigma) for 1 h. The surface of the array was washed with sterile PBS by pipetting several times, and fibronectin absorbed on the interwell region was swept carefully using a cotton swab soaked in acetone. A suspension of cells was diluted to 5×10^5 cells/mL in culture media, and 20 μL of the cell suspension was pipetted onto a set of arrays (45 × 45 microwells) and left to settle down into the microwells by gravitational force. After 10 min, residual cells on the surface between the microwells were removed by aspiration and moderate washing with the culture media. The resulting microwell array containing deposited cells was submerged in a reservoir of culture media and incubated for 6 h at 37 °C (5% CO_2) to allow attachment of cells. The percentage of occupancy and the number of cells per microwell were estimated by analyzing approximately 400 microwells under viewing fields of arrays with a 10× lens (Eclipse TS100, Nikon).

Cell Viability. Cell viability was determined using a LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular probes). Cells were stained with calcein AM according to the manufacturer's manual. Fluorescence images were obtained using a fluorescence microscope (Eeclipse ME 600, Nikon) with a B-2A filter (ex. 450−490 nm).

Microengraving and Analysis of rhEPO Secreted in a Microwell Array. The ultrathin nitrocellulose-coated glass slides (PATH slides, GenTel Bioscience) were modified with mouse antihuman EPO monoclonal antibody (R&D systems) and used to capture rhEPO secreted by single cells in the microwells. A solution of antihuman EPO monoclonal antibody (0.5 mg/mL in PBS buffer, pH 7.4) was loaded onto the surface of the slides and incubated at room temperature for 2 h in a humidity chamber (75% humidity). After incubation, the slides were immersed in TBST containing 1% bovine serum albumin (BSA, Sigma) for blocking at 4 °C for overnight. The slides were washed with deionized water immediately before use. The cell-loaded microwell array was washed with fresh culture media, followed by vacuum aspiration while tilting the array to remove excess medium on the surface of the array. For microengraving, the capture glass slide was placed on the microwell array containing cells, pressed with appropriate weight, and incubated for 1 h at 37 °C (5% CO_2). After incubation, the glass slide was removed from the microwell arrays and washed with TBST and deionized water. The slide was treated with Cy3-α-EPO (10 μg/mL) and subsequently with Cy5- MAA (100 μg/mL) using the same procedure as used in the probing of rhEPO isoforms described above. The resulting slide was thoroughly washed with TBST and deionized water, dried with nitrogen gas, and scanned with a GenePix 4100A scanner (Molecular Devices). The fluores-

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cence images were analyzed using the GenePix Pro 6.0 software (Molecular Devices). Background intensities were subtracted using median values measured for the empty wells of each subarray. The spots showing obvious defects were excluded from analysis.

Sorting and Selection of Single Cells by Statistical Analysis of Fluorescent Spots. For the selection of single cells, the intensities of Cy3-α-EPO and Cy5-MAA measured from the fluorescent spots in the glass slide were statistically analyzed. On the basis of the results with the rhEPO isoforms (Figure 2), we reasoned that the fluorescence intensity of Cy3-α-EPO was proportional to the number of rhEPO molecules secreted in a microwell and that the Cy5-MAA intensity reflects the total number of sialic acid molecules, which is dependent on both the sialic acid content per EPO molecule and the number of EPO molecules secreted in a microwell. In order to generate a normal distribution, the florescence intensities of the two probes on individual spots were converted to a logarithmic scale. Accordingly, the fluorescence intensity of Cy5-MAA, which reflects the total sialic acid content of rhEPO in a microwell, can be correlated to the Cy3-α-EPO intensity using the following relationship.

$$z_i = \alpha + \beta x_i + \varepsilon_i \qquad (i = 1, 2,...,n)$$

where z_i and x_i are the logarithmic fluorescence intensities of Cy5-MAA and Cy3-α-EPO on spot i in a slide, respectively. The slope β is the rate of change in logarithmic fluorescence intensity of Cy5-MAA for a unit change in logarithmic Cy3-α-EPO intensity, while α is the intercept. ε_i is the error term which is normally and independently distributed with zero mean and common variance δ^2. The estimates, $\hat{\alpha}$ and $\hat{\beta}$, are calculated using least-squares estimation of the logarithmic fluorescence intensities of Cy5-MAA against those of Cy3-α-EPO for all fluorescent spots.[25] Thus, the average degree of sialic acid content per EPO molecule produced by a single cell in a microwell i, y_i, can be simply expressed as the following equation,

$$y_i = \frac{z_i - \hat{\alpha}}{x_i}$$

Individual spots in the glass slide were analyzed according to the above equations and plotted in the $X-Y$ plane. Individual cells were clustered into four subgroups (namely, subgroup-1, first quadrant; subgroup-2, second quadrant; subgroup-3, third quadrant; subgroup-4, fourth quadrant) by the mean value of logarithmic Cy3-α-EPO intensity and the mean ratio of logarithmic Cy5-MAA intensity over logarithmic Cy3-α-EPO intensity. A mahalanobis distance, namely, the statistical distance from the average to each point in the $X-Y$ plane, was calculated by definition.[26] On the basis of the mahalanobis distance of an individual spot, corresponding cells from each subgroup were selected.

Retrieval and Expansion of Single Cells. Selected cells were manually retrieved from the corresponding microwells using a micropipet that had a tip diameter of 50 μm. Micropipets were drawn using a Flamming/Brown micropipet puller (Sutter Instrument). To retrieve the cells, the array of microwells was positioned under the 10× lens of an inverted microscope (DMI 3000 B, Leica) and treated with the culture media containing 10% trypsin (TrypLE Express, Gibco). The micropipet tip was positioned over a cell-containing microwell, and the media was pulled out from the microwell by capillary force. The culture media containing a detached cell in the micropipet were transferred into a 96 well plate with 200 μL of the media supplemented with 10% d-FBS. Deposition of the cells into a 96 well plate was visually verified. Retrieved cells were expanded to approximately 10^6 cells in 75 cm^2 T-flasks according to standard procedures and stored at −70 °C for banking.

Expression and Purification of rhEPO. Frozen cells were thawed and subcultured every 3 days for long-term culture. For the production of rhEPO, exponentially growing cells were seeded at a concentration of 3×10^6 cells in 175 cm^2 T-flasks and incubated for 3 days. The culture media were replaced with serum-free media (CHO-S-SFMII, Gibco) to remove serum from the culture supernatant and incubated for 2 days. The culture supernatant containing rhEPO was filtered through a 0.45 μm membrane filter (Minisart, Sartorius stedim), concentrated, and dialyzed with PBS by ultrafiltration (Amicon Ultra, Millipore). The rhEPO was purified using immune-affinity chromatography with CNBr-activated sepharose 4B (Amersham Bioscience) conjugated with antihuman EPO monoclonal antibody (R&D systems). Purified rhEPO was concentrated, lyophilized, and stored at −20 °C until analysis. To determine the specific productivity of rhEPO secreted by a single cell, exponentially growing cells were seeded at a density of 1×10^4 cells per well in a 6 well plate and incubated at 37 °C (5% CO_2). The culture media in each well were sequentially sampled every 12 h and stored at −20 °C until the assay was conducted. The number of cells in each well was estimated with respect to culture time using a hematocytometer after trypsinization.

Biochemical Analysis. The amount of rhEPO was assayed using enzyme linked immunosorbent assay (ELISA). Briefly, the microtiter plate wells were coated with mouse antihuman EPO monoclonal antibody (1 μg/mL in sodium carbonate (pH 9.0), R&D systems) for 1 h at 37 °C and treated with blocking buffer (2% BSA in TBST) for 1 h at room temperature. Rabbit anti-EPO IgG (1 μg/mL in TBST with 0.3% BSA, Sigma) and goat antirabbit IgG (H + L)−HRP conjugate (0.5 μg/mL in TBST with 0.3% BSA, Bio-Rad) were used for the immunoassay. The color was developed by treatment with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma), and the optical density of the reaction solution was measured at 655 nm using a spectrophotometer (Infinite M200, Tecan). The productivity is defined as the amount of rhEPO produced by a single cell per day. The concentration of rhEPO was estimated from the standard curve obtained with purified rhEPO, and the specific EPO productivity was calculated by plotting the EPO concentrations against the time integral values of the viable cell growth curve as described elsewhere.[27]

The sialic acid content of the purified rhEPO was determined using the *o*-phenylenediamine-2HCl (OPD) method as described elsewhere.[28] Briefly, the sialic acid released from the purified EPO under a mild acidic condition was derivatized with OPD (Sigma)

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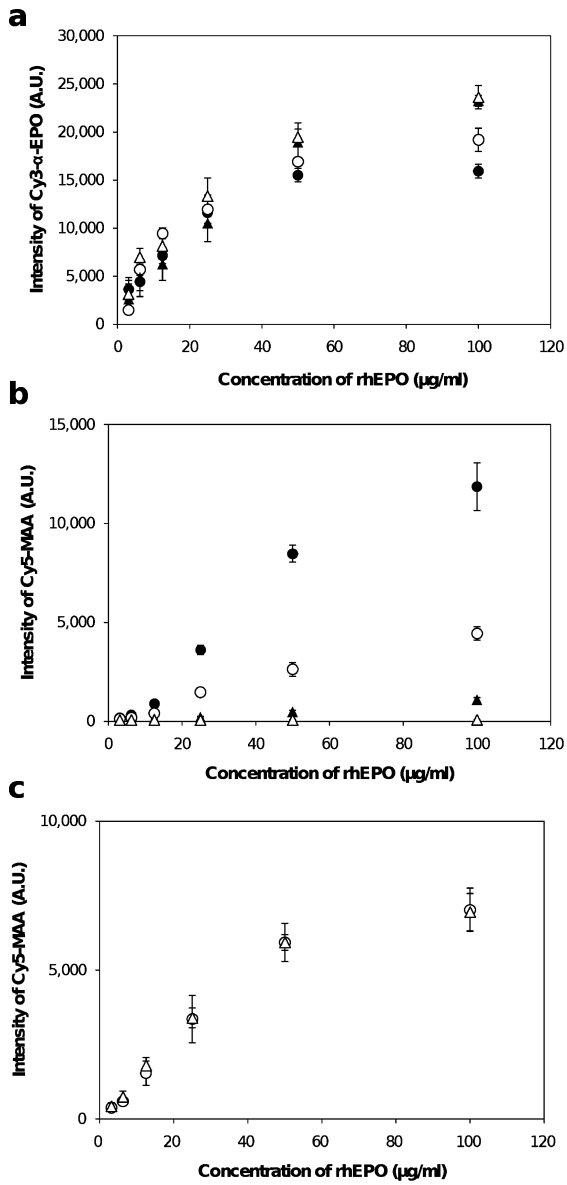

Figure 2. Changes in the fluorescence intensities of rhEPO isoforms spotted on a glass slide as a function of protein concentration when probed with Cy3-α-EPO and/or Cy5-MAA. Isofrom-1 bears the almost fully sialylated glycoform, and sialylation degree of rhEPO decreases from isoform-1 to isoform-4. Symbols are as follows: isoform-1 (●), isoform-2 (○), isoform-3 (▲), and isoform-4 (△). (a) Changes in the fluorescence intensities of the spots probed with Cy3-α-EPO. (b) Changes in the fluorescence intensities of the spots when probed with Cy5-MAA. (c) Changes in the fluorescence intensities of Cy5-MAA for isoform-1 by dual probing (○) with Cy3-α-EPO and Cy5-MAA and by a single probing (△) with Cy5-MAA.

and analyzed by HPLC with a C18-reversed phase column (Shimpack CLC-ODS, Shimadzu). The fluorescence of derivatives was detected with a fluorescence detector (474, Waters). The excitation and emission wavelength were 230 and 425 nm, respectively.

RESULTS AND DISCUSSION

Analysis of rhEPO Spotted on a Glass Slide by Dual Probing. It has been shown that CHO cell-derived glycoproteins have only α-2,3-linked sialic acid at the terminal end.[29] We reasoned that the degree of sialylation of rhEPO produced by CHO cells can be probed using *Maakia Amurensis* agglutinin (MAA) which is a sialyllactosamine (α-NeuAc-(2-3)β-Gal(1-4)β-GlcNAc/Glc)-specific lectin.[30,31] We first tested whether the amount and sialylation degree of rhEPO can simultaneously be probed with Cy3-labeled polyclonal anti-EPO antibody (Cy3-α-EPO) and Cy5-labeled *Maakia Amurensis* agglutinin (Cy5-MAA). For this, the rhEPO isoforms with different pI values were produced from almost fully sialylated rhEPO (Figure S-1, Supporting Information), spotted on a slide at various concentrations, and probed with both Cy3-α-EPO and Cy5-MAA (Figure S-2, Supporting Information). The Cy3-α-EPO intensity was almost the same for all isoforms, showing a linear increase with rhEPO concentration up to 50 μg/mL (Figure 2a). A further increase in the rhEPO concentration resulted in saturation and some deviation in the fluorescence intensity. In the case of the Cy5-MAA intensity, the increase in the fluorescence intensity was different for each isoform with varying degrees of sialylation (Figure 2b). Isoform-1 bearing the almost fully sialylated glycoform showed the most significant increase in Cy5-MAA intensity. As for isoforms-3 and -4, which had a very low degree of sialic acid content, a negligible increase in the Cy5-MAA intensity was observed even with increasing protein concentration. On the other hand, the increase in the Cy5-MAA intensities of isoforms-1 and -2 was distinct compared to that of either isoform-3 or -4 at the same protein concentration. This result strongly supports that the fluorescence intensity of Cy5-MAA can be correlated to the sialic acid content of rhEPO. In order to check the possibility of steric hindrance between α-EPO and MAA for binding to rhEPO, we compared the fluorescence intensities of Cy5-MAA between a single probing with Cy5-MAA and dual probing with both Cy3-α-EPO and Cy5-MAA (Figure 2c). In these experiments, very little difference in the fluorescence intensities of Cy5-MAA was observed, which indicates that steric hindrance between Cy3-α-EPO and Cy5-MAA was negligible. Collectively, it is clear that the amount and sialylation degree of rhEPO can be simultaneously probed using Cy3-α-EPO and Cy5-MAA.

Microengraving and Analysis of rhEPO Secreted by Individual CHO Cells in Microwells. To test the feasibility of the dual probing for analysis of rhEPO secreted by individual cells in microwells, rhEPO-producing SCST3 cells were deposited on the surface of a PDMS microwell array. The number of cells deposited per microwell might be influenced by various factors including the concentration of cells, settling time, and the size of a microwell. Of these different factors, we investigated the effect of the microwell size, and tested microwells with different diameters: 25, 30, and 40 μm. The microwell depth and interwell distance were fixed at 35 and 100 μm, respectively, regardless of its diameter. As a result, microwells with a diameter of 25 and 30 μm showed a similar occupancy ranging from one to two cells per microwell, and the occupancy was approximately 65% of the total microwells; however, the microwells with a 30 μm diameter displayed a slightly higher occupancy than those with 25 μm in diameter (Figure S-3a, Supporting Information). In the case of microwells with a 40 μm diameter, the occupied portion of microwells increased, but at the same time, the number of

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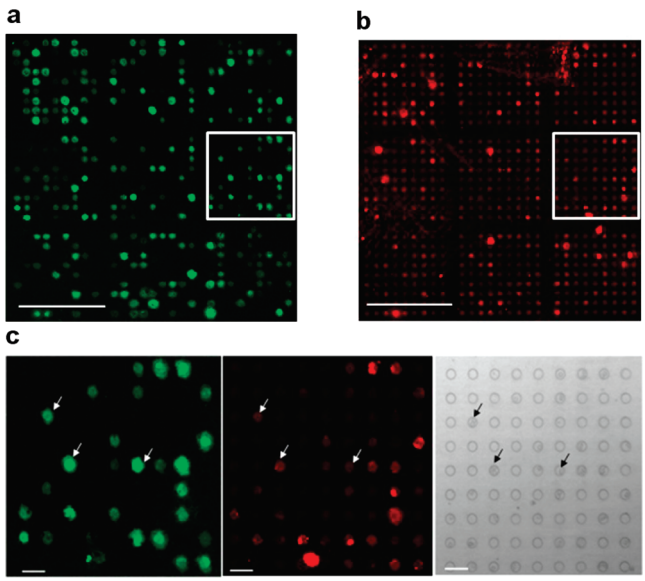

Figure 3. Fluorescence images of a glass slide prepared from a cell microwell array. rhEPO secreted by individual cells in microwells was captured using a glass slide coated with anti-EPO monoclonal antibody, probed with Cy3-α-EPO (green) and Cy5-MAA (red), and scanned using a fluorescence scanner. Scale bars, 1 mm. (a) Fluorescence image when scanned for Cy3-α-EPO. (b) Fluorescence image when scanned for Cy5-MAA. (c) Enlarged images of the region indicated by a white box in a and b and a phase-contrast image of the corresponding microwells. The arrows indicate the fluorescent spots and corresponding cells contained in microwells. Scale bars, 100 μm.

microwells containing more than two cells also significantly increased. On the basis of these results, we chose microwells with a 30 μm diameter for further experiments. Cells confined in the microwells with a size of 30 μm in diameter could be clearly observed using a phase-contrast microscope (Figure S-3b, Supporting Information). A PDMS slab of 6 mm ×6 mm contained 2025 microwells, and the volume of each microwell with a 30 μm diameter was estimated to be 25 pL.

The cell-containing microwell array was submerged in a reservoir of culture media for 6 h, followed by mild washing with fresh serum-free media three times. During washing, release or detachment of cells from the microwells was not observed. For microengraving, the microwell array was dewetted and placed in contact with a glass slide coated with anti-EPO monoclonal antibody, followed by compression with an appropriate weight and incubation for 1 h. Most of the cells in the microwells were found to remain alive after incubation for 1 h even without a supplement of fresh media (Figure S-4, Supporting Information). The resulting slide was probed with Cy3-α-EPO and Cy5-MAA and scanned using a scanner to correlate their fluorescence intensities with the amount and terminal sialic acid content of secreted rhEPO in individual microwells. Figure 3a,b shows typical fluorescence images of the slide comprising the arrays of secreted rhEPO, and spots emitting distinct Cy3-α-EPO and Cy5-MAA signals were found. In addition, there were significant variations in the Cy3-α-EPO and Cy5-MAA intensities from spot to spot. Interestingly, the spots exhibiting distinct Cy3-α-EPO and Cy5-MAA signals matched well with the cell-containing microwells (Figure 3c).

Variations in the fluorescence intensities of Cy3-α-EPO and Cy5-MAA on the slide clearly indicate that the amount and sialylation degree of rhEPO produced by individual cells in microwells were different and can be analyzed using the dual probing method. The levels of fluorescence signals from the spots corresponding to empty microwells were negligible compared to those from occupied microwells. This result reflects the fact that nonspecific binding of fluorescently labeled proteins (Cy3-α-EPO and Cy5-MAA) was insignificant on the antibody-coated slide and empty microwells. Some spots were found to emit an unusually high Cy5 signal, which seems to result from the lysis of cells released from the microwells, and these were excluded from further analysis. The fluorescence intensity of Cy5-MAA was relatively low compared to that of Cy3-α-EPO; this seems to be due to a lower binding affinity of MAA for rhEPO than antibody.[31]

Sorting of Individual Cells. In order to select a single cell producing rhEPO with a high sialylation degree, we first sorted individual rhEPO-producing SCST3 cells into four subgroups based on the statistical analysis of the fluorescence intensities of the two probes on spots as described in the Experimental Section. Of approximately 1000 spots displaying fluorescence signals in each experiment, around 150−200 spots were screened for sorting by taking into account the single-cell occupancy and fluorescence intensities of the corresponding microwells. The intensity of Cy3-α-EPO reflects the amount of secreted rhEPO, and the fluorescence intensity of Cy5-MAA is a measure of the total sialic acid content of the secreted rhEPO. Accordingly, the total number of sialic acids in a microwell can be estimated by multiplying the average number of sialic acid per EPO molecule by the total number of EPO molecules secreted in the microwell. Consequently, the ratio of the Cy5-MAA intensity to the Cy3-α-EPO intensity represents the sialylation degree of each EPO molecule. As can be seen in Figure 4a, sorted individual cells displayed variations in logarithmic fluorescence intensity of Cy3-α-EPO and the ratio of the logarithmic fluorescence intensities between Cy3-α-EPO and Cy5-MAA. Cells in the respective subgroup represent a typical characteristic in terms of the amount and sialylation degree of produced rhEPO. For example, cells in subgroup-2 reflect those producing rhEPO with a sialylation degree higher than the average level, but the amount of rhEPO produced is lower than the mean. Meanwhile, cells in subgroup-4 are thought to produce rhEPO with a lower sialylation degree than the mean level, but the production level is above the average.

Selection, Retrieval, and Expansion of Single Cells. To validate the sorting of individual cells based on statistical analysis of the dual probing, representative cells (ten cells from each subgroup) were selected, retrieved, and expanded. Cells were easily detached from the microwells by trypsin treatment, and the average survival rate of the retrieved cells ranged from 60 to 70%. Individual cells were expanded to around 10^6 cells in 75 cm^2 T-flasks according to standard procedures and stored at −70 °C for banking. Of the stored cells, four cell lines (sc1-29, sc1-18, sc1-122, and sc1-2) from respective subgroup-1 to -4 were grown for the production of rhEPO. At the same time, cells were seeded at 1×10^4 cells per well in a 6 well plate to determine the specific productivity of rhEPO secreted by a single cell as described in the Experimental Section. As shown in Table 1, the productivities of four cell lines (sc1-29 to sc1-2) were estimated

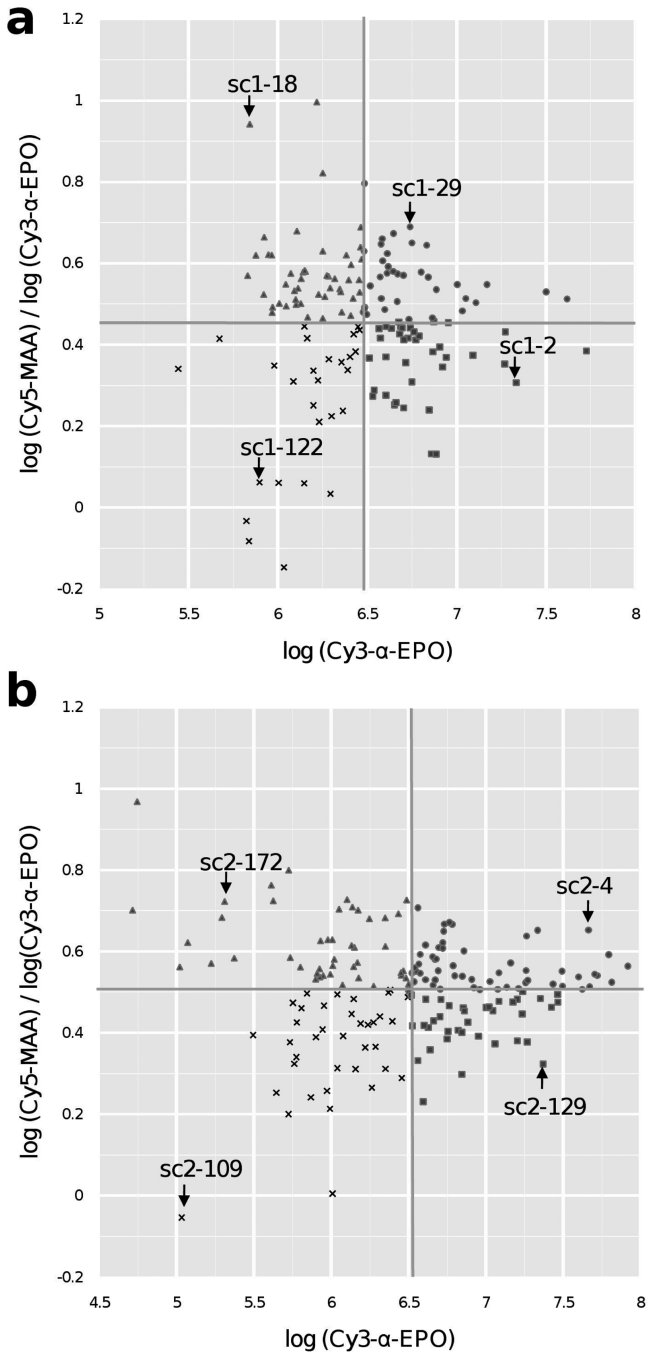

Figure 4. Sorting of rhEPO-producing cells derived from different maternal cell lines. On the basis of the statistical analysis of the dual probing, corresponding individual cells were clustered into four subgroups by the mean value of logarithmic Cy3-α-EPO intensity and the mean ratio of logarithmic Cy5-MAA intensity over logarithmic Cy3-α-EPO intensity. The *X* axis represents the logarithmic intensity of Cy3-α-EPO, and the *Y* axis is the ratio of logarithmic Cy5-MAA intensity to logarithmic Cy3-α-EPO intensity. Arrows indicate the cell lines expanded for the production of rhEPO at each round of selection. Symbols are as follows: subgroup-1 (●), subgroup-2 (▲), subgroup-3 (×), and subgroup-4 (■). (a) Sorting of rhEPO-producing cells derived from SCST3 cell line. (b) Sorting of rhEPO-producing cells derived from sc1-18 cell line.

to be 4.3 ± 0.4, 3.4 ± 0.1, 3.2 ± 0.5, and 4.8 ± 0.7 pg/cell/day, respectively, which indicates that cell lines showing high Cy3-α-EPO intensities (sc1-29 and sc1-2) produced rhEPO at a relatively high rate compared to those with lower intensities (sc1-18 and sc1-122). The average numbers of sialic acids per rhEPO secreted by the four cell lines (sc1-29 to sc1-2) were determined to be about 8.7 ± 0.5, 9.0 ± 0.6, 8.2 ± 0.3, and 7.8 ± 0.5, respectively (Table 1). The sc1-18 cell line selected from subgroup-2 produced rhEPO with the highest sialic acid content, while its production rate of rhEPO was lower than the other cell lines. The glycosylation efficiency is generally known to increase with a reduced translational rate of the protein,[29] and there seems to be compromise between the sialylation degree and production rate of rhEPO at the single-cell level. This result shows that the sc1-29 to sc1-2 cell lines represented the typical features of cells in their respective subgroup in terms of the sialylation degree and amount of produced rhEPO, even though there were some deviations, validating the sorting of individual cells based on the statistical analysis of the fluorescence intensities of two probes on individual spots. Interestingly, selected cell lines were found to produce rhEPO with an average number of sialic acid higher or slightly lower than the starting cell line (SCST3). The average number of sialic acid of rhEPO produced by the SCST3 cell line was about 8.0 ± 0.3. This result seems to be due to the low avidity of the lectin used for the probing of the sialic acid content. The spots emitting distinct fluorescence intensities of both probes were subjected to analysis, and cells producing rhEPO with a low sialylation degree were primarily excluded from sorting. Consequently, the sorted cells were likely to produce rhEPO with a relatively high degree of sialic acid content. From a clinical point-of-view, the terminal sialic acid content is crucial to the therapeutic efficacy of rhEPO, because the plasma half-life of EPO is known to be greatly affected by the terminal sialic acid content.[5,7] In this regard, we chose the sc1-18 cell line for the next round of selection.

Table 1. Productivities and Sialic Acid Contents of rhEPO by Selected Cell Lines Derived from the SCST3 and sc1-18, Respectively[a]

maternal cell line	subgroup	cell line	productivity (pg rhEPO/cell/day)	sialic acid per EPO (nmol/nmole)
SCST3	1	sc1-29	4.3 ± 0.4	8.7 ± 0.5
	2	sc1-18	3.4 ± 0.1	9.0 ± 0.6
	3	sc1-122	3.2 ± 0.5	8.2 ± 0.3
	4	sc1-2	4.8 ± 0.7	7.8 ± 0.5
sc1-18	1	sc2-4	4.0 ± 0.3	9.1 ± 0.3
	2	sc2-172	3.4 ± 0.1	9.8 ± 0.1
	3	sc2-109	3.2 ± 0.3	8.5 ± 0.2
	4	sc2-129	4.4 ± 0.1	8.5 ± 0.1

[a] Values represent means ± SD of two independent experiments.

Repeated Round of Single Cell Selection. In order to select a single cell producing rhEPO with increased sialylation degree, we repeated the same selection procedure starting with the sc1-18 cell line. On the basis of the statistical analysis of the fluorescent spots on a glass slide, approximately 200 cells were sorted into four subgroups (Figure 4b), and ten cells from each subgroup were selected, retrieved, and expanded for banking. Similarly, representative cells from each subgroup were thawed and grown further for the production of rhEPO. As a result, the productivities of rhEPO by four selected cell lines were determined to be 4.0 ± 0.3, 3.4 ± 0.1, 3.2 ± 0.3, and 4.4 ± 0.1 pg/cell/day, respectively (Table 1). Both sc2-4 and sc2-129 cell lines showing high Cy3-α-EPO signals exhibited higher productivity compared to those with

lower signals, which represents the typical features of the cells in their respective subgroup. As a whole, four cell lines selected in the second round exhibited almost the same productivities as those selected in the first round. As for the sialic acid content, the sc2-172 cell line selected from subgroup-2 produced rhEPO with the highest sialic acid content, and the average number of sialic acid per rhEPO molecule reached 9.8 ± 0.1 (Table 1). Consequently, the average number of sialic acid per rhEPO molecule increased by about 25% compared to that of the starting cell line SCST3 through repeated selection of single cells using the same procedure. As observed in the first round, selected cell lines in the second round produced rhEPO with sialic acid content higher or lower than the starting cell line (sc1-18). This result seems to reflect intraclonal variation of glycosylation in a clonal population. Recently, it was reported that there exists intraclonal heterogeneity among protein-secreting cells even though cells were derived from a single parental clone.[32,33] Many factors are known to be involved in protein glycosylation, and the variation in glycosylation remains to be elucidated.

CONCLUSION

We have demonstrated that the array-based analysis of secreted glycoproteins enables the rapid and efficient selection of a single cell producing a glycoprotein with desired glycosylation. Our approach provides a simple and easy way of analyzing the single cells based on multiplexed interrogation of proteins secreted by individual cells in a microwell array, thus offering several advantages over conventional methods. First, the detection strategy allows the rapid selection of a single cell producing a glycoprotein with specific glycosylation without detailed characterization of the glycoprotein at each round of clonal selection. Second, the approach based on the dual probing significantly reduces the number of cells to be tested, since individual cells leading to distinct fluorescence signals above certain levels are subjected to sorting. A variety of therapeutic glycoproteins including antibodies, blood factors, interferons, and some hormones are currently used and under development. More than 100 lectins specific for different carbohydrate moieties are known,[34] and other kinds of glycan-specific probes such as aptamers and peptides might become available. Thus, this method can be widely used in the clonal selection for the production of other glycoproteins with specific glycosylation. Furthermore, the present method should be useful for investigating the heterogeneity in cell populations based on multiplexed glycan analysis of secreted proteins.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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