Technical Advance

Development of a Frozen Cell Array as a High-Throughput Approach for Cell-Based Analysis

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Recent advances in molecular biology, human genetics, and functional genomics tremendously increase the number of molecular targets available for potential therapeutic and diagnostic use. To complement DNA array data, cost-efficient high-throughput technologies providing reliable information at the protein level need to be developed. Here we describe the generation of a frozen cell array that required the use of single cell suspensions and could serve various applications such as the analysis of specific antibody or ligand binding to a large panel of different cell types. As an example, binding of an anti-human epithelial cell adhesion molecule monoclonal antibody to 24 different cell lines has been analyzed using the cell array and compared to the data generated by fluorescence-activated cell sorting. The reliability and flexibility of our frozen cell array technology is compatible with the needs of high-throughput screening for drug discovery and target validation. (*Am J Pathol* 2002, 161:787–797)

Publication of the human genome sequence and related data-mining information are facilitating the identification of increasing numbers of targets for drug discovery and target validation. DNA microarray technology currently enables the selection of candidate molecules as potential drug targets. This approach provides important information concerning gene expression and potential genetic alterations that could be confirmed using other molecular analyses such as RNA *in situ* hybridization. These molecular technologies in general, however, do not provide critical information about the ultimate effector, the expressed proteins.

Therefore, additional technologies have been developed to evaluate molecular candidates at the protein

level. One such case is the recently developed tissue microarray technology that allows for the rapid high-throughput profiling of normal and tumor tissue specimens. In addition to allowing the investigator to assess histomorphology, tissue microarrays could be used to analyze the expression of molecules at the DNA, mRNA, and protein levels.¹ Potential applications for tissue microarrays span a broad range and include the analysis of the frequency of molecular alterations in large numbers of tumors, exploration of tumor progression, identification of predictive or prognostic factors, and validation of newly discovered genes as diagnostic and therapeutic targets at a speed comparable to DNA microarrays.¹ At the protein level, the main limitation of the tissue microarray technology stems from the fact that specimens are fixed and paraffin-embedded. These conditions are not optimal for a significant number of antibodies or ligands that only bind to native epitopes or binding sites and therefore require the use of fresh or frozen biological material. Considering the technical challenge represented by the development of frozen tissue microarrays as well as the limited availability of fresh or frozen tissues, fluorescence-activated cell sorting (FACS) provides an alternative method for analysis of antibody/antigen or ligand/receptor binding in a biological context using primary cells or cell lines.² Large screening efforts involving the analysis of numerous antibodies or proteins using one or more cell types, however, are time consuming and difficult to perform using FACS. Various techniques including laser-scanning cytometry³ and imaging,⁴ cellular biosensor,⁵ immunobiosensor with engineered molecular recognition,⁶ and lab-chip microfluidic systems (see the Caliper homepage on the World Wide Web, URL:<http://www.calipertech.com/> and the ACLARA Biosciences homepage on World Wide Web URL:<http://www.aclara.com/>) have been recently developed to achieve a higher throughput. These approaches are powerful screening tools for the analysis of different cell types as well as the analysis of various molecules at the same time. For high-throughput screening, however, these technologies

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could be labor intensive and expensive considering that they involve living cells that have to be maintained in culture. This requirement could also introduce biases in data if changes in cell genotype and phenotype occurred throughout time *in vitro*. In addition, these strategies require complex and expensive equipment that could also limit their usage.

Despite these recent technological progresses, a need for simple, reliable, and cost-efficient approaches that allow high-throughput parallel cell-based analysis still exists. This need prompted us to develop a new technology called a frozen cell array that allows for the analysis of a large number of cell types in a single experiment. This approach takes advantage of the cryopreservation of cells and provides a broad application base including antibody- and ligand-binding studies in a well-preserved bioenvironment. In this study, we describe the construction of our frozen cell array and present data relative to the human epithelial cell adhesion molecule (Ep-CAM),⁷ a 40-kd glycoprotein that has a major morphoregulatory function, relevant to tissue development, carcinogenesis, and tumor progression. The frozen cell array results for 24 different cell types were compared to the FACS data generated using the same anti-Ep-CAM monoclonal antibody.

Materials and Methods

Cell Lines

The frozen cell array was developed using various normal and tumor cell lines. Normal cells included: human iliac artery endothelial cells (HIAECs), human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells from lung (HMVECs), normal human dermal fibroblasts (NHDFs), human coronary artery smooth muscle cells (CASMCS), and neonatal normal human epidermal keratinocytes (NHEKs) purchased from BioWhittaker/Clonetics (San Diego, CA) and the bovine kidney glomerular endothelial cell (BKGEs) purchased from VEC Technologies, Inc. (Rensselaer, NY, USA). These cells were carried in vendor recommended complete media at 37°C in a humidified atmosphere of 5% CO₂ in air. Tumor cells included: human colorectal carcinoma cell lines COLO205, SW620, HCT116; human prostate carcinoma cell lines PC3, DU145; human breast carcinoma cell lines SkBr3, MCF7, BT474; human malignant melanoma cell line A375; human rhabdomyosarcoma cell line A673; human neuroglioma cell line U87MG; human lung carcinoma cell line SKMES1; human liver carcinoma cell lines HepG2, Hep3b; T- and B-cell leukemia cell lines Jurkat and Bjab, respectively, were purchased from American Type Culture Collection (Manassas, VA). The tumorigenic NRP154 cell line derived from normal adult rat prostate was a gift from Dr. Gerald Cunha (University of California at San Francisco, San Francisco, CA). SW620, HCT116, DU145, SkBr3, BT474, A375, A673, U87MG, SKMES1, HepG2, Hep3b, Jurkat, and Bjab were carried in RPMI 1640 supplemented with L-glutamine (2 mmol/L) and 10%

fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂ in air. BKGE cells were carried in RPMI 1640 supplemented with L-glutamine (2 mmol/L) and 10% adult calf serum in the same conditions. COLO205 cells grown in suspension, NRP154, PC3, and MCF7 were carried at 37°C in a humidified atmosphere of 5% CO₂/95% air in F12/Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

FACS Analysis

Adherent cells were detached from tissue culture flasks in the presence of 0.5 mmol/L ethylenediaminetetraacetic acid for 15 to 20 minutes at room temperature and centrifuged at 1000 rpm for 5 minutes. Detached cell suspension cells were washed once with phosphate-buffered saline (PBS) before resuspension in PBS containing 1% bovine serum albumin (BSA). The cells were counted, adjusted to 10⁶ cells/ml, and 1 ml of cells was incubated in PBS/1% BSA for 30 minutes at 4°C. Subsequently, these cells were incubated in the presence or absence of 0.5 µg of mouse anti-human Ep-CAM fluorescein-conjugated monoclonal antibody (Biomedica Corp., Foster City, CA) in 1 ml of PBS/1% BSA. After a 1-hour incubation at 4°C, cells were washed, resuspended in 1 ml of PBS/1% BSA and analyzed using a flow cytometer Coulter Epics XL-MCL (Beckman Coulter, Inc., Fullerton, CA).

Construction of the Frozen Cell Array

To generate an array of wells in optimal cutting temperature medium (OCT), we custom made a tool called an arrayer (Figure 1). This arrayer consisted of 25 blunts (Pharmaseal, Glendale, CA) heat-sealed and glued with epoxy (ITWDevcon, Danvers, MA) in a block of Plexiglas. Small pieces of metal and epoxy were used to plug each blunt (40-mm long, 1.2-mm diameter). The final arrayer, containing 25 pins equally spaced (less than 1.4 mm) in a 12 × 12-mm area, was used to create an array of wells in OCT medium (Figure 2). After immersion of the arrayer in glycerol (Figure 2A) and subsequently in OCT in disposable embedding molds (22 × 30 mm, 20-mm deep) (VWR, San Francisco, CA) (Figure 2B), the complete setup was submerged in isopentane at -160°C (Figure 2C). After 3 to 5 minutes in a cryobath, extraction of the arrayer from the OCT block, yielded an array of wells at least 20-mm deep (Figure 2D). This array was stored at -70°C until the loading of various cell lines into the wells. Adherent cells were detached from tissue culture flasks in the presence of 0.5 mmol/L of ethylenediaminetetraacetic acid for 15 to 20 minutes at room temperature, centrifuged at 1000 rpm for 5 minutes, and washed in PBS at 4°C. Suspension cells (COLO205, Jurkat, and Bjab) were directly washed in PBS at 4°C. The cell number was determined using a particle count analyzer (Coulter Z2; Beckman Coulter, Miami, FL) and cells were resuspended in 70 to 150 µl of cold PBS based on their availability at the time of loading to obtain a highly concentrated cell suspension. The final density of the cell suspensions loaded into the array were as follows: BKGEs, 92.9 × 10⁶ cells/ml; COLO205, 259.3 × 10⁶ cells/ml; HCT116, 82.5 × 10⁶ cells/ml; SW620, 95 × 10⁶ cells/ml; PC3, 56.4 × 10⁶ cells/ml; DU145, 52.9 × 10⁶ cells/ml;

Figure 1. Custom built arrayer. A 12 × 12-mm arrayer has been constructed using 25 blunts (40-mm long, 1.2-mm diameter), heat-sealed, and glued with epoxy onto a block of Plexiglas. The unsealed blunt extremities were subsequently plugged using small pieces of metal and epoxy.

NRP154, 74.1×10^6 cells/ml; HIAECs, 60.4×10^6 cells/ml; HUVECs, 65×10^6 cells/ml; HMVECs, 124×10^6 cells/ml; NHDFs, 36.1×10^6 cells/ml; CASCs, 45.1×10^6 cells/ml; NHEKs, 42.3×10^6 cells/ml; A375, 79.4×10^6 cells/ml; SkBr3, 53.3×10^6 cells/ml; MCF7, 26×10^6 cells/ml; BT474, 72.4×10^6 cells/ml; A673, 172.7×10^6 cells/ml; HepG2, 66.6×10^6 cells/ml; Hep3b, 57.4×10^6 cells/ml; Jurkat, 614.4×10^6 cells/ml; Bjab, 335.5×10^6 cells/ml; SKMES, 73.9×10^6 cells/ml; and U87MG, 86.6×10^6 cells/ml. The OCT block containing the array was placed onto a box filled with dry ice at room temperature and less than 100 μ l of cell suspensions maintained at 4°C were loaded into the wells of the array using 1-ml syringes with 22G $^{1/2}$ (0.7 mm × 40 mm) needles (Becton Dickinson, Franklin Lakes, NJ) (Figure 2E). Position A1 on the array was loaded with Trypan blue stain 0.4% (Life Technolo-

gies, Inc., Grand Island, NY) and used as a landmark. After loading the different cells, the frozen cell array was stored at -70°C before sectioning. Sections, 6- or 12- μ m-thick, were cut on a cryostat and laid on precleaned microscope slides (75 × 25 mm, 0.96 to 1.09-mm thick) (Baxter Diagnostic Inc., Deerfield, MI) (Figure 2F). Each slide contained two sections of the cell array (<12 mm²) with each spot measuring a diameter less than 1.1 mm and spaced by less than 1.4 mm apart (Figure 2G). The frozen cell array slides were subsequently stored at -70°C.

Immunocytochemistry Procedure

Frozen cell array slides were air-dried at room temperature for at least 3 hours before fixation in acetone for 5 minutes

Figure 2. Construction of the frozen cell array. **A:** The arrayer's pins were first immersed in glycerol to subsequently facilitate the extraction of the arrayer from the frozen block of OCT. **B:** The arrayer was plunged in OCT in disposable embedding molds. **C:** The complete setup was immersed in the cryobath. **D:** An array of wells was created into the frozen block of OCT after extraction of the arrayer. **E:** Loading of highly concentrated cell suspensions into the wells of the frozen array placed onto a box filled with dry ice. **F:** Sections, 6- or 12- μ m thick, were cut on a cryostat and collected on precleaned microscope slides. **G:** Final format of the cell array. Two sections of the cell array containing 25 spots were placed on each microscope slide.

and overnight air-drying. Endogenous immunoglobulin-binding sites were blocked with PBS/1% BSA (30 minutes) and cell array slides were overlaid for 1 hour at room temperature with PBS/1% BSA containing 0.5 $\mu\text{g/ml}$ of mouse anti-human Ep-CAM fluorescein-conjugated monoclonal antibody (Biomedica Corp.). A background control was run in the absence of labeled antibody. After several rinses in PBS, sections were treated with Hoechst (100 $\mu\text{g/ml}$) (Molecular Probes, Eugene, OR) for 2 minutes and rinsed again before mounting with mounting medium (Vectashield; Vector, Burlingame, CA) and cover glass (22 mm², N°1; Corning, Garden Grove, NY). Cell array slides were protected from light and dust until analysis with either a fluorescence microscope ($\times 40$ or $\times 400$ magnification) (Eclipse E800; Nikon, Melville, USA) linked to a charge-coupled device (CCD) camera (Photometric CH350; Roper Scientific, Tucson, AZ), a Typhoon 8600 scanner (Molecular Dynamics, Sunnyvale, CA), or a custom-built CCD camera (white light source; Genentech Inc., South San Francisco, CA). No signal was observed in the control without mouse anti-human Ep-CAM fluorescein-conjugated monoclonal antibody. The Hoechst fluorescence was analyzed only with the microscope setup.

Computer Analysis of the Cell Array Images

Cell array image files acquired either with the fluorescence microscope setup with the QED CCD camera stand-alone software (QED Imaging Inc., Pittsburgh, PA), the Typhoon 8600 scanner or the CCD camera were first processed using Photoshop software (Adobe Photoshop 4.0; Adobe System Inc., San Jose, CA). Separate images obtained using the same capture parameters on the fluorescence microscope ($\times 40$ magnification) were used to rebuild the complete image of the cell array in Photoshop. Images of the complete cell array acquired directly using the Typhoon 8600 or after reconstruction in the case of the microscope or the CCD camera were converted to inverted gray scale, 8 bits/channels TIFF files and analyzed using the Phoretix Array² software (Nonlinear USA Inc., Durham, NC). After positioning and cropping cell array images to the software, a grid was designed to fit the 25 spots of the array and spot properties were defined to tightly circle the spot area. Among the different outputs available in the Phoretix Array software, we chose spot volume for this study. We neglected other outputs available in the software. The volume of a spot is calculated as the total intensity of the spot after subtraction of the background intensity. For the background subtraction, the spot surface minimum algorithm was chosen as a local method. This algorithm takes the lowest intensity value inside the spot and applies the value as the background for all pixels in the spot. Background calculations are therefore made independently for each spot. Under these conditions, the signal quantification is not affected by the distribution of pixel intensities across a spot. Quantification of the signal for each cell array spot was compared to FACS results.

Results

Cell Array Analysis of the Anti-Human Ep-CAM Monoclonal Antibody (mAb) Binding to COLO205 Cells

To evaluate the performance of our cell array as a tool to study the expression of specific antigens on various cells, a frozen cell array was initially generated as described in Materials and Methods using only the human colorectal carcinoma cell line COLO205. This COLO205 cell array was subsequently used to analyze the expression of the epithelial glycoprotein Ep-CAM (also known as KSA, EGP, GA733-2 antigen)^{8–10} using an anti-human Ep-CAM fluorescein-conjugated mAb. Encoded by the GA733-2 gene this antigen originally described as colon tumor antigen¹¹ is also expressed on the basolateral cell surface in most human simple, pseudo-stratified, and transitional epithelia and is often present at the cell surface of human carcinoma cells.⁷ Cell array sections of 6- or 12- μm thickness were used for Ep-CAM immunocytochemistry. As expected, the Hoechst nuclear fluorescent staining observed using the microscope for the COLO205 cell spot from the 12- μm section was stronger than the one observed on the 6- μm section (Figure 3, C and A, respectively). Quantification of the Hoechst fluorescence using the Phoretix Array² software revealed a 43% signal increase on the 12- μm section compared to the 6- μm section (Figure 3E). Similar results were observed for the Ep-CAM-specific fluorescein signal (Figure 3, B as compared to D). The quantification of the fluorescein signal revealed a 27.3% signal increase on the 12- μm section compared to the 6- μm section (Figure 3F). These data strongly suggest that the thickness of the cell array section is an important parameter that could be modulated to increase the fluorescent signal. The fact that in this study the increase of the section thickness did not result in an increase of the background suggests that this parameter might also be used to increase the sensitivity of the technique. Subsequently, 12- μm sections were used for the rest of the study.

FACS Analysis of the Anti-Human Ep-CAM mAb Binding to 24 Different Cell Lines

To compare our frozen cell array to an already well-established technology, we analyzed the binding of the anti-human Ep-CAM fluorescein-conjugated mAb to 24 different cell lines by FACS (Figure 4). A strong positive signal was observed for the human colon tumor cell lines COLO205 (A3), HCT116 (A4), and SW620 (A5); the human breast carcinoma cell lines SkBr3 (D1), MCF7 (D2), and BT474 (D3); and the human liver carcinoma cell lines HepG2 (D5) and Hep3b (E1). A moderate EP-CAM expression was observed for the human and rat prostate

Figure 3. Effect of the cell array section thickness on the fluorescence signal. **A** and **C**: Hoechst staining (DNA) of a 6- and 12-μm-thick COLO205 cell array section, respectively. **B** and **D**: Ep-CAM staining of a 6- and 12-μm-thick COLO205 cell array section, respectively, using an anti-human Ep-CAM fluorescein-conjugated mAb. **E** and **F**: Quantification of the Hoechst and fluorescein signal on a 6- or 12-μm-thick COLO205 cell array section, respectively.

tumor cell lines DU145 (B2) and NRP154 (B3), respectively, and the neonatal NHEKs (C4). Low to negative signal was observed for the human lung carcinoma cell line SKMES1 (E4); the NHDFs (C2); the bovine endothelial cells BKGES (A1), used as a negative cell control; the prostate tumor cell line PC3 (B1); the human normal endothelial cell lines HIAEC (B4), HUVEC (B5), HMVEC (C1); the human normal smooth muscle cells CASMC (C3); the human malignant melanoma cell line A375 (C5); the human rhabdomyosarcoma cell line A673 (D4); the leukemia cell lines Jurkat (E2) and Bjab (E3); and the human neuroglioma cell line U87MG (E5). Our results on Ep-CAM expression in various cell lines are in agreement with the expression pattern previously described for this cell surface molecule in normal and pathological conditions.¹¹

Cell Array Analysis of the Anti-Human Ep-CAM mAb Binding to 24 Different Cell Lines Using Different Image Acquisition Systems

The different cell lines used for the analysis of Ep-CAM expression by FACS were also used on the same day and at the same passages to generate a frozen cell array according to the protocol described in Materials and Methods. Frozen cell array sections of 12-μm thickness were cut and used to perform an immunocytochemistry study using an anti-human Ep-CAM fluorescein-conju-

gated mAb. Hoechst nuclear staining was applied to the cell array sections and the corresponding fluorescent staining captured using the fluorescent microscope setup (Figure 5A). The observation of the Hoechst signal revealed the spatial heterogeneity of several array spots such as spots B5 (HUVEC) or D4 (A673). Because the different cell types present on the array were not loaded at the same cell density, the Hoechst signal intensity appeared different on each spot. Indeed, the spot corresponding to the Jurkat cells (E2) loaded at the highest density (614.4×10^6 cells/ml) appeared the brightest and the spots loaded with a cell suspension at a density lower than 50×10^6 cells/ml appeared the faintest [NHDF (C2), CASMC (C3), NHEK (C4), and MCF7 (D2)]. The fluorescein signal (Ep-CAM) was captured using the fluorescent microscope setup (Figure 5B), a Typhoon 8600 scanner (Figure 5C) or a white light source CCD camera (Figure 5D). Similar results were generated regardless of the setup used to acquire the fluorescein cell array image. The strongest fluorescein signal (Ep-CAM) was observed for the COLO205 (A3), HCT116 (A4), SW620 (A5), HepG3 (E1), and SkBr3 (D1) cell lines. Detectable fluorescein signal was also observed for the DU145 (B2), NRP154 (B3), MCF7 (D2), BT474 (D3), and HepG2 (D5) cell lines. A very weak signal could also be seen for the CASMC (C3), HUVEC (B5), and U87MG (E5) cell lines. No signal was observed in the other cell lines present on

Figure 4. Binding of an anti-human Ep-CAM fluorescein-conjugated mAb to various cell lines analyzed by FACS. BKGEs (**A2**); human colorectal carcinoma cell lines COLO205 (**A3**), HCT116 (**A4**), and SW620 (**A5**); human prostate carcinoma cell lines PC3 (**B1**) and DU145 (**B2**); rat prostate tumorigenic cell line NRP154 (**B3**); HIAECs (**B4**); HUVECs (**B5**); lung HMVECs (**C1**); NHDFs (**C2**); human CASMCs (**C3**); neonatal NHEKs (**C4**); human malignant melanoma cell line A375 (**C5**); human breast carcinoma cell lines SkBr3 (**D1**), MCF7 (**D2**), and BT474 (**D3**); human rhabdomyosarcoma cell line A673 (**D4**); human liver carcinoma cell lines HepG2 (**D5**) and Hep3b (**E1**); T- and B-cell leukemia cell lines Jurkat (**E2**) and Bjab (**E3**), respectively; human lung carcinoma cell line SKMES1 (**E4**); and human neuroglioma cell line U87MG (**E5**) were incubated in the absence (*thin line*) or presence (*thick line*) of an anti-human Ep-CAM fluorescein-conjugated monoclonal antibody and analyzed by FACS.

the cell array: BKGE (**A2**), PC3 (**B1**), HIAEC (**B4**), HMVEC (**C1**), NHDF (**C2**), NHEK (**C4**), A375 (**C5**), A673 (**D4**), Jurkat (**E2**), Bjab (**E3**), and SKMES1 (**E4**).

Comparative Study of the Ep-CAM Expression in Different Cell Lines Using FACS or Cell Array Analysis

The cell array images were analyzed using the Phoretix Array² analysis software. Through this software the fluorescein and the Hoechst signal intensity was quantified for each spot. The different cells present on the cell array were ranked based on their Ep-CAM expression level according to the FACS data or the cell array data generated using either the fluorescence microscope, a Typhoon 8600 scanner or a white light source CCD camera (Table 1, Figure 6). For the FACS analysis the cell lines

were ranked for Ep-CAM expression based on the mean fluorescence observed in the presence (Ab) or in absence (Ctr) of the anti-human Ep-CAM fluorescein-conjugated mAb. The fluorescein signal acquired using the fluorescence microscope was used to rank the cell lines for Ep-CAM expression either directly or after its normalization by the Hoechst signal (DNA content). Finally, the fluorescein signal acquired using the scanner or the CCD camera was used directly to rank the different cell lines for Ep-CAM expression. As presented in Table 1 and Figure 6, the comparative Ep-CAM expression profile generated using our cell array appeared very similar to the one observed by FACS. Indeed, the 10 cell lines presenting the highest Ep-CAM expression levels as determined by FACS are similar to the one determined using our cell array. Although some differences were observed for the detailed ranking depending on the data

Figure 5. Capture of the cell array fluorescent signal using different image acquisition systems. **A:** Hoechst fluorescent staining was analyzed for each spot using the same capture parameters on the fluorescence microscope and separate images were used to rebuild the complete cell array. **B:** Anti-human Ep-CAM fluorescein-conjugated mAb binding to the cell array analyzed using the same capture parameters on the fluorescence microscope. Separate images were used to rebuild the complete cell array. **C and D:** Anti-human Ep-CAM fluorescein-conjugated mAb binding to the cell array was analyzed using a Typhoon 8600 scanner or a white-light source CCD camera, respectively. Remarkably, the same array slide was used for the fluorescence microscopy and white-light source CCD camera analysis, whereas a different slide was used for the typhoon 8600 scanner analysis. The different cell lines were positioned onto the array as follows: **A2**, BKG; **A3**, COLO205; **A4**, HCT116; **A5**, SW620; **B1**, PC3; **B2**, DU145; **B3**, NRP154; **B4**, HIAEC; **B5**, HUVEC; **C1**, HMVEC; **C2**, NHDF; **C3**, CASMC; **C4**, NHEK; **C5**, A375; **D1**, SkBr3; **D2**, MCF7; **D3**, BT474; **D4**, A673; **D5**, HepG2; **E1**, Hep3b; **E2**, Jurkat; **E3**, Bjab; **E4**, SKMES1; and **E5**, U87MG. Original magnifications, $\times 40$.

acquisition and analysis processes, the cells could be similarly classified as strong (COLO205, HCT116, SW620, SkBr3, MCF7, BT474, HepG2, and Hep3b), moderate (DU145, NRP154), or low and negative (SKMES1, NHDF, BKGE, PC3, HIAEC, HUVEC, HMVEC, CASMC, A375, A673, Jurkat, Bjab, U87MG) Ep-CAM expressors. The only exception was the NHEK cell line that had a higher Ep-CAM expression level in the FACS analysis compared to the array analysis. It is important to notice that the cell classification based on their Ep-CAM expres-

sion was generally similar whether or not normalized to the Hoechst signal. However, the normalization of the Ep-CAM fluorescein signal to the DNA Hoechst signal could improve the correlation between FACS and cell array results (for example see MCF7 classification in Table 1). In addition, although we used a different array slide for the Typhoon 8600 scanner analysis, the data obtained still appeared very similar to the one generated from another slide using the fluorescent microscope or the white light source CCD camera. This demonstrates

Table 1. Comparison of the FACS and the Cell Array Data Acquired Either with the Microscope Setup, a Typhoon 8600 Scanner, or a White Light Source CCD Camera

Array position	Cell	Frozen cell array analysis										
		FACS analysis		Microscope			Ratio	Ranking	Scanner		CCD	
		(Ab/Ctr)-1	Ranking	FITC	Ranking	Hoechst			FITC	Ranking	FITC	Ranking
A2	BKGE	0.0000	15	0	22	74239	0.0000	20	0	20	0	26
A3	colo205	117.5714	1	542811	1	481274	1.1279	2	77431	1	783243	1
A4	HCT116	41.8571	3	416666	2	368796	1.1298	1	42621	2	408458	3
A5	SW620	64.8571	2	327044	3	313890	1.0419	3	18992	4	134121	5
B1	PC3	0.0000	15	493	13	151923	0.0032	15	46	17	15770	12
B2	DU145	4.0000	11	18888	10	241427	0.0782	10	2563	8	40149	7
B3	NRP154	5.8571	9	26098	9	303313	0.0860	9	4739	7	44215	6
B4	HIAEC	0.0000	15	2571	11	44347	0.0580	11	0	20	6529	13
B5	HUVEC	0.0000	15	249	15	270108	0.0009	17	378	13	6001	14
C1	HMVEC	0.2000	14	43	17	328260	0.0001	18	185	14	1657	22
C2	NHDF	0.0000	15	42	18	9166	0.0046	14	0	20	3371	19
C3	CASMC	0.0000	15	341	14	32804	0.0104	12	818	12	17258	11
C4	NHEK	4.5000	10	4	20	31618	0.0001	18	0	20	3285	20
C5	A375	1.8750	12	4	21	183621	0.0000	19	113	15	855	25
D1	SkBr3	13.2857	6	80832	5	235842	0.3427	6	12072	5	137542	4
D2	MCF7	36.6667	4	28328	8	55737	0.5082	4	1544	10	29717	9
D3	BT474	10.0000	7	34030	7	85093	0.3999	5	1570	9	33271	8
D4	A673	0.0000	15	0	23	138486	0.0000	20	0	20	4209	17
D5	HepG2	8.0000	8	43500	6	163610	0.2659	8	5131	6	17750	10
E1	Hep3b	34.6000	5	81729	4	238971	0.3420	7	33110	3	415421	2
E2	Jurkat	0.0000	15	0	24	472655	0.0000	20	9	19	1215	23
E3	Bjab	0.0000	15	16	19	370906	0.0000	19	14	18	1192	24
E4	SKMES1	0.8750	13	231	16	185182	0.0012	16	48	16	2246	21
E5	U87MG	0.0000	15	586	12	77352	0.0076	13	1136	11	4437	15

The different cell lines were ranked based on their Ep-CAM expression levels. For the FACS analysis, the cells were ranked based on the mean fluorescence observed in presence (Ab) or in absence (Ctr) of the anti-human Ep-CAM fluorescein (FITC)-conjugated MAb (Ab/Ctr)-1). For the cell array analysis, the cells were ranked based on the direct fluorescein signal only or after its normalization to the Hoechst signal (DNA content) in the case the images were capture using the microscope setup.

the reproducibility of our method at least when we use different slides made from serial sections. These results validate our cell array approach as a direct semiquantitative analysis method.

Qualitative Analysis of the Ep-CAM Expression in Different Cell Lines Using the Cell Array

As a result of the efficient processing of the cells on the frozen OCT array and potentially the presence of glycerol mixed with OCT at the periphery of each well that could also act as cryoprotectant, the morphology of the cells on the array appeared well preserved. Therefore the immunofluorescence staining was analyzed at a high microscopic magnification ($\times 400$) for each spot present on the cell array. The high-magnification images captured using different parameters depending on the staining intensity ($\times 400$ magnification) were used to rebuilt the entire cell array after superimposition of the fluorescein (Ep-CAM) and Hoechst (DNA) signal as shown in Figure 7A. In agreement with the previously described cell surface localization of the Ep-CAM antigen,¹¹ a moderate to strong specific membrane staining was observed for the Ep-CAM-positive cell lines (COLO205 (A3), HCT116 (A4), SW620 (A5), SkBr3 (D1), MCF7 (D2), BT474 (D3), HepG2 (D5), Hep3b (E1), DU145 (B2), NRP154 (B3), and NHEK (C4). Low specific cell surface staining or no staining was observed for the cells analyzed as weak [SKMES1 (E4), (NHDF) (C2)] or negative [BKGE (A1), PC3 (B1), HIAEC

(B4), HUVEC (B5), HMVEC (C1), CASMC (C3), A375 (C5), A673 (D4), Jurkat (E2), Bjab (E3), and U87MG (E5)] Ep-CAM expressors. Interestingly, a strong cell surface staining was observed for the neonatal NHEKs (C4) analyzed as Ep-CAM-positive by FACS. In addition to confirm the cell surface localization of Ep-CAM for some of the cell lines present on the array, the high-magnification analysis of the spot also revealed differences in the nature of the staining. This is the case for the liver carcinoma cell line Hep3b in which the staining appeared granular compared to the COLO205 cells in which it is more homogenous (Figure 7B).

Discussion

The recent completion of the sequencing of the human genome and other organisms has provided a tremendous amount of information to the scientific community. To make sense of this information, high-efficiency analytical technologies are required. To date, DNA microarrays have been the choice approach for analyzing the expression of thousands of genes in a single hybridization. Based on this technology, biotechnology and pharmaceutical industries have developed strategies to identify candidate molecules for potential therapeutic applications. DNA microarrays, however, do not provide information about the protein itself and therefore additional methods that allow for the validation of potential drug

Figure 6. Graphic representation of the Ep-CAM expression level analyzed by FACS (**A**) or the cell array signal analyzed either with the microscope setup (**B**, FITC only; **C**, FITC normalized by Hoechst signal), the scanner (**D**), or the CCD camera (**E**).

target in a given tissue or cells are needed. Although the different approaches currently used provide valuable information, disadvantages such as low-throughput, poor protein preservation, or high cost, limit their use.

In this study, we describe the development of a frozen cell array that could be used as a multiparameter high-throughput screening method. Using a custom-made arrayer, we generated an array of wells in a frozen block of OCT. These wells were subsequently loaded with different types of normal or tumor cell lines and sections of the cell array were generated.

Our cell array technology represents a major improvement compared to other methods previously described.^{12,13} The frozen cell array is easy to develop and cell array slides can be made in a few minutes in contrast to hours using other approaches.¹² Because cells are directly loaded and instantaneously frozen on the array, they do not require fixation¹² or extensive handling before loading.¹³ Consequently, the cell morphology is well preserved on the array sections. The direct molding of the array into the OCT block using our arrayer approach avoids the use of any kind of tubes as described elsewhere.¹³ This is certainly a major advantage considering that the cell array must subsequently be cut into very thin sections and that the presence of a solid material can make this task laborious. According to our own experience, sectioning through a solid material such as plastic tubes using the cryostat, generated plastic fibers as well as rings of plastic that were not stable on the microscope slide. In addition, the presence of such plastic material represents a major source of potential background. The molding of an array of wells into the frozen block of OCT also provides significant flexibility. After molding, the frozen array could be stored indefinitely at -70°C and dif-

ferent cells could be loaded onto the array individually or several at a time depending on their availability. This cell loading process is useful because cells have different growth rates, because the cell lines are being developed and are not ready to be loaded on the array, or as cells are being transformed with different DNA constructs that may not be ready simultaneously. This offers a strong advantage over other technologies (Li R, Mather JP: Cell arrays and the uses thereof. International patent application 1999, PCT/US00/34010),¹² that require the development of an entire cell array at one time. The use of the custom built arrayer also allows the development of frozen cell arrays containing a large number of wells in a given area. Recently, we developed a frozen array that could contain up to 50 different cell types per microscope slide.

As an example, we used our cell array technology to study the binding of an anti-human Ep-CAM fluorescein-conjugated mAb to various cell lines. First, we demonstrated that in the case of this Ep-CAM immunocytochemistry analysis the increase of the thickness of the cell array sections resulted in an increase of the fluorescent signal without increase of the background. This result strongly suggests that this parameter could potentially be used to increase the sensitivity of the technique. This parameter in addition to the density of the cell suspension loaded onto the array could be independently optimized depending of the cell array application. In this study, 12- μm -thick sections were used and because the optimal cell density for this application was initially unknown, the different cell types were loaded into the array at various cell densities depending on their availabilities at the time of the array loading. Under these conditions, our cell array immunofluorescence analysis appeared to be comparable to FACS analysis and more accurate for the

Figure 7. Qualitative analysis of the staining on the cell array treated with the anti-Ep-CAM fluorescein-conjugated mAb. **A:** Reconstruction of the cell array using high-magnification images after superimposition of the fluorescein (Ep-CAM) and Hoechst (DNA) signal. **B:** Immunofluorescence staining with the anti-human Ep-CAM fluorescein conjugated mAb on the Hep3b and COLO205 cells. The different cell lines were positioned onto the array as follow: **A2**, BKGE; **A3**, COLO205; **A4**, HCT116; **A5**, SW620; **B1**, PC3; **B2**, DU145; **B3**, NRP154; **B4**, HIAEC; **B5**, HUVEC; **C1**, HMVEC; **C2**, NHDF; **C3**, CASMC; **C4**, NHEK; **C5**, A375; **D1**, SkBr3; **D2**, MCF7; **D3**, BT474; **D4**, A673; **D5**, HepG2; **E1**, Hep3b; **E2**, Jurkat; **E3**, Bjab; **E4**, SKMES1; and **E5**, U87MG.

spots where the cells have been loaded with a cell suspension at a density higher than 50×10^6 cells/ml that is equivalent to at least 2000 cells/spot. This probably explains why the FACS and cell array data relative to the expression of Ep-CAM did not correlate for the NHEK cells, because these cells were loaded onto the array at a cell density lower than 50×10^6 cells/ml. The subsequent qualitative analysis of the fluorescence staining on the cell array, however, confirmed the expression of Ep-CAM at the cell surface of the neonatal keratinocytes. It is also important to notice that although the cells were loaded at different cell densities on the frozen array, the normalization of the Ep-CAM-specific fluorescent signal to the DNA-specific fluorescent signal did not dramatically change the results of the comparative expression study performed for the different cell lines. The cells detected as strong, moderate, low, or negative Ep-CAM expressors are the same independently of the technology used to acquire the array image (microscope, Typhoon 8600 scanner, or a custom-built white light source CCD camera). This is probably because of the way we analyzed the data through the Phoretix Array² software. Indeed, the measurement of the volume of a spot involves the calculation of the spot threshold. This threshold is defined as the ratio of the raw volume to the maximum volume, which is a function of the number of pixels per spot. Therefore, Phoretix array² software data processing appeared to correct for cell density differences using the parameters described in Materials and Methods. We also demonstrated some reproducibility of our method because we obtained similar data using different array slides made with serial sections of the array block. This is particularly important because spot failures could happen over a large number of slides. Indeed, with deeper sections in the block, we observed the absence of a couple of array spots. Manual loading of the cells probably accounts for such results because gaps could be generated inside the frozen cell array block. We currently focus our efforts in developing an automation system to optimize the loading of the cells and improve the consistency of our approach. Meanwhile, we load cells in duplicate onto the new arrays in development.

Based on this data, we conclude that our cell array technology could easily be used to provide reliable protein expression data that meet the needs of a broad protein expression screening even though cells were loaded on the array at various densities ($>50 \times 10^6$ cells/ml). In addition, this technology could also be used for quantitative applications involving antibodies or ligand-binding proteins, DNA, and RNA measurement if the different cells present on the array are loaded at the same density.

Finally, if we consider the good preservation of the cells on the array, this approach potentially provides additional information regarding the cellular localization of a given signal applying different immunocytochemistry and histochemistry methods. Again, the thickness of the cell array section could be modulated to accommodate applications as complex as confocal microscopy.

In summary, we have developed a low-cost frozen cell array containing 24 different cell lines and demonstrated its reliability for comparative, qualitative, and semiquantitative

antibody-binding studies using an anti-human Ep-CAM fluorescein-conjugated mAb. This technology could be used for a large number of other applications that we are currently investigating including the detection and the quantification of ligand-binding-specific proteins, DNA, and RNA in different cell types concurrently. Pilot experiments have been successfully performed for actin RNA *in situ* hybridization as well as IGF1 ligand binding. The reliability and flexibility of this low-cost high-throughput technology, combined with the ease of development and use, make the frozen cell array a qualitative and semiquantitative screening tool of choice for drug discovery and target validation. Considering the successful development of our frozen cell array technology, we are currently investigating the possibility to adapt our method to primary human tumor samples to develop frozen tissue microarrays.

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