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# Quantitative proteomic profiling of breast cancers using a multiplexed microfluidic platform for immunohistochemistry and immunocytochemistry

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# ABSTRACT

This paper describes a multiplexed microfluidic immunohistochemistry (IHC)/immunocytochemistry (ICC) platform for quantitative proteomic profiling in breast cancer samples. Proteomic profiling via ICC was examined for four breast cancer cell lines (AU-565, HCC70, MCF-7, and SK-BR-3). The microfluidic device enabled 20 ICC assays on a biological specimen at the same time and a 16-fold decrease in time consumption, and could be used to quantitatively compare the expression level of each biomarker. The immunohistochemical staining from the microfluidic system showed an accurate localization of protein and comparable quality to that of the conventional IHC method. Although AU-565 and SK-BR-3 cell lines were classified by luminal subtype and adenocarcinomas and were derived from the same patient, weak p63 expression was seen only in SK-BR-3. The HCC70 cell line showed a triple-negative (estrogen receptor-negative/progesterone receptor-negative/human epidermal growth factor receptor 2-negative) phenotype and showed only cytokeratin 5 expression, a representative basal/myoepithelial cell marker. To demonstrate the applicability of the system to clinical samples for proteomic profiling, we were also able to apply this platform to human breast cancer tissue. This result indicates that the microfluidic IHC/ ICC platform is useful for accurate histopathological diagnoses using numerous specific biomarkers simultaneously, facilitating the individualization of cancer therapy.

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# 1. Introduction

Breast cancer, one of the most frequent and deadly cancers in women, has been recognized as a heterogeneous disease in terms of natural history, genetic alteration, histopathological features, gene expression profile, and response to treatment in individual patients [1–4]. Its heterogeneity indicates that individualized, tailored therapy is essential for appropriate and optimal treatment. Through advances in genomics, proteomics, and molecular pathology, many potential biomarkers have been demonstrated to have clinical value [5]. Thus, in the near future, it is expected that various types of molecular therapies for cancer patients will gradually increase, moving away from a simple and widespread prescription towards tumor-specific administration and therapy [6].

Recently, a molecular taxonomy, using DNA microarrays, has been proposed for the classification of breast cancers [7–9]. Five subtypes (luminal A, luminal B, basal, ERBB2-over-expression, normal-like types) have been identified based on different patterns of gene expression, underlying DNA copy number alterations, and associated clinical outcomes. This approach has a significant impact on breast cancer management and understanding the genotypic and phenotypic properties of cancers. Although genotypic classification supports personalized medicine, significant changes in gene expression might not be reflected to the level of protein expression or practical function [10]. Additionally, some discrepancies have been shown between analyses of mRNA and protein levels [11]. Thus, for a better understanding of breast cancers and their refined classification, proteomic profiling is also important.

Immunocytochemistry (ICC) has been a widely used and established immunological detection method. Additionally, the use of immunohistochemical staining has been a major part of the routine diagnostic procedure in various malignancies, and recent studies have reported a relationship between immunohistochemistry (IHC) profiles of various types of breast carcinomas and molecular taxonomic classification (the five subtypes based on DNA microarray profiling) [12–15]. Because IHC/ICC is cost effective and



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**Fig. 1.** Schematic of the microfluidic massive IHC/ICC device. The device was designed to screen 10 biomarkers on a sample at the same time. Inset shows the cross-sectional (a–a') view of the microvalve consisting of two layers: fluidic channel (bottom, blue) and control channel (top, red).

suitable for routine practice in individual patients and is applicable to formalin-fixed and paraffin-embedded tissue fragments and identification of *in situ* protein signatures, it is one of the optimal proteomic technologies in pathological diagnoses [16]. Thus, proteomic profiling via IHC is a promising method of refined subclassification of complex tumor phenotypes and tailored therapy in practice with a quantitative fashion. Unfortunately, an appropriate technology for proteomic profiling via the IHC/ICC method has rarely been demonstrated.

Here, we have developed a massively multiplexed and quantitative IHC/ICC platform using microfluidics technology and have applied it to breast cancer cell lines. Breast cancer cell lines have many advantages in the investigation of breast cancer pathobiology, and screening, and the characterization of novel therapeutics [6,17,18]. Additionally, they are able to support an unlimited and readily handled source and exhibit purity of cancerous epithelia (free from contamination of stromal cells). In this study, 10 biomarkers, known to be predictive and prognostic indicators in breast cancers, were examined on a cell block, and 20 IHC/ICC

#### Table 1

The procedure of the microfluidic massive IHC/ICC method.<sup>a</sup>

No.	Reagent	Time
1	Deparaffinization	$4 \min \times 2$
2	Rehydration process	$1 \text{ min} \times 5$
3	Washing using deionized (DI) water	$1 \min \times 4$
4	Heat-induced epitope retrieval	20 min
5	Cooling	5 min
6	Hydrogen peroxide blocking	10 min
7	Washing using TBS tween buffer	1 min
8	Blocking solution	20 min
9	Primary antibody treatment	15 min
10	Washing using TBS tween buffer	1 min
11	Secondary antibody treatment	20 min
12	Washing using TBS tween buffer	1 min
13	Streptavidin-HRP	20 min
14	Washing using DI water	1 min
15	DAB	5 min
16	Washing using DI water	1 min
17	Mayer's hematoxylin	3 min
18	Washing using DI water	1 min
19	Dehydration	$1 \text{ min} \times 5$
20	Xylene	$4 \text{ min} \times 2$
	Total consumed time	154 min

<sup>a</sup> Most procedure was conducted in off-chip and the treatment of primary antibodies was performed by the microfluidic device. assays were performed simultaneously and compared quantitatively. The availability of quantitative proteomic profiling via the IHC method was also addressed for human breast cancer tissue.

#### 2. Materials and methods

#### 2.1. Design and fabrication of the microfluidic IHC/ICC device

The device was designed to screen 10 biomarkers on a breast cancer cell block. Because there are many reservoirs for biomarkers, fluid flow was driven by with-drawal of a syringe pump connected to the outlet. The diameter of the biomarker reservoirs was 1.5 mm, and they were filled via individual syringes. The reaction channels were 300 µm in width, and the gap between reaction channels was 50 µm (Fig. 1). Fluidic resistance was considered for equivalent flow rates of individual reaction channels. For rectangular channels, the channel flow rate is expressed as  $Q = \Delta P/R$ , where the  $\Delta P$  is the pressure drop and *R* is the fluidic resistance. The resistance *R* is expressed as follows:

$$R = f \operatorname{Re} \frac{\mu L}{2D_{\rm s}^2 A} \tag{1}$$

$$D_h = \frac{2wh}{w+h} \tag{2}$$

 $fRe = 96 \left( 1 - 1.3553\alpha + 1.9467\alpha^2 - 1.7012\alpha^3 + 0.9564\alpha^4 - 0.2537\alpha^5 \right)$ (3)

where *w*, *h*, and *L* are the width, height, and characteristic length of a microfluidic channel, respectively;  $\mu$  is the viscosity of a fluid; *A* is the cross-sectioned area of a microfluidic channel; and  $\alpha$  is the aspect ratio [19,20]. In this study, the planar area for each reaction channel was fixed.

Microvalves were required not only to fill the biomarkers into individual reservoirs, but also to react 10 biomarkers with a target sample simultaneously. Thus, rounded channels were fabricated for microvalves, and rectangular channels were created to form even fluid profiles in terms of the width of the reaction channels [21]. The fluidic channel mold of the device was fabricated by two-step multilayer soft lithography. To construct rectangular reaction channels, a negative photoresist, SU-8 2025 (MicroChem Corp., Newton, MA), was spincoated to make a 60- µm thickness on a bare silicon wafer, patterned by UV light exposure. After developing the wafer, a positive photoresist, AZ 9260 (MicroChemicals GmbH, Ulm, Germany), was spincoated to make a 20-  $\mu m$  thickness on the wafer, creating a round-shaped remnant fluidic channel. It was exposed to UV light with a fluidic channel mask and developed by an AZ photoresist developer (AZ 400K (1:3.5)). The fabricated mold was reflowed by a heating process in the sequence of 70  $^\circ$ C (5 min), 85 °C (5 min), 95 °C (5 min), and 110 °C (30 min). The fluidic channels, except the reaction channels, were transformed into round shapes. The control channel mold of the device was fabricated by conventional SU-8 photolithography. The fluidic channel mold was spincoated with polydimethylsiloxane (PDMS) to make a thin membrane and cured at 70 °C for 1 h. The membrane including fluidic channels was aligned and bonded with the control channel layer by an O<sub>2</sub> plasma asher (270 W, 30 s).

#### 2.2. Preparation of cell blocks and tissues

Four breast cancer cell lines, AU-565, HCC70, MCF-7, and SK-BR-3, were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The preparation of cell blocks and tissues was described in our previous report [21]. Briefly, HCC70, MCF-7, and AU-565 were maintained in RPMI-1640 medium, and SK-BR-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU mL<sup>-1</sup> penicillin, and

#### Table 2

Breast cancer markers used in this study and their condition.

Protein	Company	Clone	Dilution factor	Working concentration in the microfluidic platform
S-H	DAKO	SMMS-1	×200	×0.25
CK5	Novocastra	XM26	$\times 50$	×0.25
CK14	Novocastra	LL002	×100	×0.25
E-ca	Transduction	36	×250	×0.25
p53	VENTANA	Bp53-11	$\times 1$	×0.25
ER	VENTANA	6F11	$\times 1$	×0.25
p63	DAKO	4A4	×50	×0.25
PR	VENTANA	1A6	$\times 1$	×0.25
HER2	DAKO	polyclonal	×1000	×0.25
Ki-67	DAKO	MIB-1	×50	×0.25



**Fig. 2.** An image of reaction channels along which fluids are flowing. (A) Each dye was flowed along the individual reaction channel without cross-contamination. Scale bar, 1 mm. (B) An image showing simultaneous elimination of solutions in reservoirs. The withdrawal flow rate was 600 μL/h and the injected amount of solution for each reservoir was 10 μL in volume. After approximately 10 min 15 s, all solutions were empty at the same time. Scale bar, 2 mm.

100 mg mL<sup>-1</sup> streptomycin. To make a cell block, the harvested cells were centrifuged, fixed in formalin, suspended in agar, and embedded in paraffin. Paraffinembedded cell blocks were sectioned at 4  $\mu$ m. Sections were mounted and baked onto positively charged slides and dried for 1 h at room temperature followed by 1 h in a convection incubator at 60 °C.

Human tissue samples from each tumor lesion were obtained from the Korea University Anam Hospital (Seoul, Korea), with the corresponding written consent provided by the patients or their relatives. This study was approved by the Institutional Review Board (IRB) at the Korea University Anam Hospital. Tissue samples from each tumor lesion (Korea University of Medicine, Korea) were fixed for 24 h in 4% neutral-buffered formalin, Bouin's fixative, acetic formalin alcohol (AFA), or 4% or 10% unbuffered formalin, 4 h in PreFer (Anatech, Battle Creek, MI) or Pen-Fix (Richard Allen Scientific; Kalamazoo, MI), or 48 h in 4% neutral-buffered formalin. When paraffin embedding procedure was completed, tumor specimens were cut into 4 µm sections and dried for 1 h at room temperature, and then incubated for 1 h in a 60 °C oven.

#### 2.3. Process of massive IHC/ICC staining using a microfluidic device

The overall procedure for massive IHC/ICC is described in Table 1. Cell blocks and tissues were de-waxed in xylene and rehydrated through a graded series of ethanol solutions (100%, 95%, 90%, 80%, and 70% ethanol). After the hydration process, a microwave antigen-retrieval technique was used and the samples were treated with a target retrieval solution, pH 9 (Dako, Denmark), for 20 min at 750 W. After cooling the sample slide, it was treated with hydrogen peroxide (LabVision, Fremont, CA) and a blocking solution. The microfluidic device was then aligned with the sample, and a reversible seal was created by pressing the device with a weight. Ten biomarkers were flowed along the individual reaction channels for 15 min. After detachment of the sample slide from the device, the sample was treated with secondary antibody, streptavidin-horseradish peroxidase (HRP), and 3,3'-diaminobenzidine tetrahydrochloride (DAB) in sequence; the Cap-Plus kit (Zymed, San Francisco, CA) was used for staining. Mayer's hematoxylin (Labvision, Fremont, CA) was used for counterstaining. Finally, cell blocks and tissues were dehydrated through a graded series of ethanol solutions (50%, 80%, 90%, 95%, and 100% ethanol), treated with xylene, and fixed with Canada balsam (Sigma-Aldrich, St. Louis, MO).

Because tissue slides stained via IHC are stored after mounting with Canada balsam, the device should be worked in a state of reversible sealing. Therefore, we created an assembler to connect a microfluidic device and a tissue/cell slice. For assembly of a device and a sample slide, the fabricated microfluidic device was first connected with an upper plate to plainly press the device from a weight, treating air plasma to make a hydrophilic surface. When a glass slide containing a cell block or tissue was mounted on a bottom plate, the upper plate was assembled with the bottom plate. The reaction channels of the device were then aligned with the sample and pressed by weight. A microvalve was manipulated by air pressure (40 kPa), and LabView software was used to control the air pressure. Ten biomarkers were flowed using a withdrawal-mode syringe pump connected to the outlet port.

#### 2.4. Predictive and prognostic biomarkers in breast cancer

Ten biological markers were used in this study, and all biomarkers were 0.25fold diluted compared with the antibody concentration of the conventional IHC method (Table 2). Estrogen receptor (ER) antibody (Ventana, Tucson, AZ) and progesterone receptor (PR) antibody (Ventana) were used in conventional methods at  $1 \times$  ready-to-use concentrations and in the microfluidic device at a dilution of 1:4. Human epidermal growth factor receptor 2 (HER2) oncoprotein antibody (Dako) was used in conventional methods at a dilution of 1:500 and in the microfluidic device at a dilution of 1:2000. Ki-67 antibody (Dako) was used in conventional methods at a dilution of 1:50 and in the microfluidic device at a dilution of 1:200. Cytokeratin 14 (CK14; Novocastra, Newcastle-upon-Tyne, UK), cytokeratin 5 (CK5; Novocastra), E-cadherin (E-ca; BD Biosciences, Franklin Lakes, NJ), p53 (Ventana), p63 (Dako), and smooth muscle myosin heavy chain (S-H; Dako) were used in the microfluidic device at dilutions of 1:400, 1:200, 1:1000, 1:4, 1:200, and 1:800, respectively.

#### 2.5. Image acquisition and analysis for quantification of the microfluidic IHC/ICC

A cell block or tissue slide stained with the microfluidic system was placed on an inverted microscope (Carl Zeiss, Germany), and images were acquired under 13,000 Lux light intensity. The acquired microscopic images were divided into three parts:



**Fig. 3.** Alignment of the massively multiplexed device with a SK-BR-3 cell block. (A) An image being aligned between the device and the cell block after the blocking process. (B) A stained image showing that the microfluidic massive ICC process was finished. All scale bars are 800 μm.



**Fig. 4.** Images of breast cancer cells stained with several biomarkers by a conventional ICC method and the microfluidic method. (A–F) Results of immunocytochemical staining conducted by the conventional method. (G–L) Immunocytochemical staining was conducted using the large-scale microfluidic IHC/ICC platform. (A, G) An image of AU-565 cells stained with HER2. (B, H) An image of MCF-7 cells stained with ER. (C, I) An image of MCF-7 cells stained with PR. (D, J) An image of HCC70 cells stained with Ki-67. (E, K) An image of SK-BR-3 cells stained with p53. (F, L) An image of HCC70 cells stained with CK5. All magnifications for the images are 400×.

staining part (SP) of a cell, non-staining part (NSP) of a cell, and background part. The three parts were classified by a Bayesian classification. The Gaussian mixture model and expectation-maximization method were used for the color distribution of each part in RGB color space [21].

To minimize variation in the image acquisition including cell density, the ratio of the staining area was calculated by SP over SP and NSP, and the staining intensity was averaged only in the SP. The value of the "expression level" was calculated by multiplying the staining ratio and the averaged intensity. At least three images were taken per reaction channel, and the average expression level for the images is presented as a representative value of immunocytochemical staining for a biomarker.

The measurement of biomarkers is fitted by a one-dimensional Gaussian mixture model to show the continuous measurement. Ten biomarkers were numbered from 1 to 10: p53, E-ca, CK14, CK5, S-H, ER, p63, PR, HER2, and Ki-67. Suppose we have measurements  $f_i$  (i = 1, 2, ..., 10). The fitted measurement at x, f(x), can be expressed by

$$f(\mathbf{x}) = \sum_{i=1}^{M} f_i B(\mathbf{x}; \mu_i, \sigma) \tag{4}$$

where the basis function  $B(x; \mu, \sigma) = \exp\{-\frac{(x-\mu)^2}{2\sigma^2}\}$  is the one-dimensional Gaussian function centered at  $\mu$  and scaled by  $\sigma$ .  $B(x; \mu, \sigma)$  is 1 at  $\mu$  and diminishes as x goes far from  $\mu$ . The center of the *i*th biomarker  $\mu_i$  is located at i-1/2 and the scaling factor is set by 0.01.

# 3. Results and discussion

# 3.1. Characterization of the microfluidic IHC/ICC platform

Since the microfluidic massive IHC/ICC platform was conducted in a reversible sealing condition, cross-contamination can be problematic. Thus, a microfluidic device without defects in the walls between reaction channels is important. To make such an intact device, approximately 5 min should pass after pouring PDMS on a fabricated mold to sufficiently fill the gaps between reaction channels. The mold was then spincoated and cured. The device worked under 8 kPa of pressure, and fluids flowed along the



Fig. 5. Image analysis of 10 biomarkers' expressions in the HCC70 cell line. (A) A picture of expressions of 10 biomarkers for the HCC70 cell line. Scale bar, 200  $\mu$ m. (B) The picture after image analysis of Fig. 5A. Scale bar, 200  $\mu$ m. (C) Magnified image of the dotted area in Fig. 5A. Scale bar, 50  $\mu$ m. (D) Magnified image of the dotted area in Fig. 5B. Only brown colors clearly remain in the image. Scale bar, 50  $\mu$ m.

individual reaction channel without cross-contamination (Fig. 2A). Additionally, because this system flowed in a withdrawal mode, it should be verified whether 10 biomarkers connected to each reaction channel flow equivalently, indicating that the flow rate is the same between biomarkers. We inserted the same amount of solution in each reservoir (10  $\mu$ L) and measured the consumption time for the solution to be eliminated. As shown in Fig. 2B, all solutions disappeared simultaneously. From these results, we could indirectly determine that the individual reaction channels had almost the same flow rate. The reproducibility of flow rate was also examined. The experiment was performed in triplicate, and the consumed time was 10 min 15 s  $\pm$  13 s at a pumping rate of 600  $\mu$ L/h withdrawal.

#### 3.2. Massive multiplexed ICC on a cell block

Before a cell block was treated with a blocking process, the microfluidic device was treated with plasma and was then ready to integrate with the sample block. After the blocking process, the device was aligned with the cell block and a weight was applied (Fig. 3A). During the aligning process, a condensed area of cells was selected to maximize the cell number stained with ICC and minimize the error of image analysis. Thus, in this case, the SK-BR-3 cell block was biased towards the right side. Air pressure was applied to the microvalve to close the channels connected biomarker reservoirs, and the 10 biomarkers were inserted into the reservoirs. When the process was finished, the microvalve was opened and all of the biomarkers simultaneously flowed via operation of a syringe pump. After treatment of biomarkers, the cell block was carefully detached from the device and the next ICC protocol was followed. Fig. 3B shows the result of massive ICC on a cell block. The pattern

of immunocytochemical staining was equivalent to the geometry of the reaction channels.

Staining quality was compared between the microfluidic massive ICC platform and conventional ICC method (Fig. 4). HER2, a cell membrane surface-bound tyrosine kinase, is localized in the plasma membrane of cells. Results showed that brown colors were expressed only in the plasma membrane of AU-565 cells by both methods (Fig. 4A and G). On the other hand, the hormone receptors (ER and PR), Ki-67, and p53 are localized in the nucleus; they were stained only in the nucleus of breast cancer cells by both methods (Fig. 4B-E and H-K). In particular, ER and PR demonstrated relatively heterogeneous staining compared with other biomarkers. Cytokeratin 5 (CK5), an indicator of the basal epithelial layer with the transcription factor p63 [22], is expressed in the cytoplasmic area of cells [23]. In this case, both methods showed proper localization of expression in HCC70 cells (Fig. 4F and L). Although the microfluidic massive ICC method used 0.25-fold diluted antibodies and the incubation time was also reduced as much as 4-fold, the staining guality was comparable to the conventional ICC method. When assuming that the general ICC process takes about 4 h for one biomarker, the microfluidic system, capable of 20 ICC assays, saved time by 16-fold. From these results, we concluded that the platform has a credible quality of immunocytochemical staining and can be used as a multiplexed immunocytochemical method to screen the expression of various biomarkers. Additionally, it is easy to use, reproducible, credible, and compatible with the conventional ICC method.

# 3.3. Proteomic profiling for breast cancer cells via the microfluidic platform

Cell line models are frequently used in breast cancer studies [18]. Molecular profiling of breast cancer cell lines has been studied



**Fig. 6.** Quantitative proteomic profiling of 10 biomarkers for 4 breast cancer cell lines by ICC. (A) A graph of quantified expression levels for 10 biomarkers in the AU-565 cell line. (B) A graph of quantified expression levels for 10 biomarkers in the HCC70 cell line. Because the cell line is a basal subtype, ER-, PR-, and HER2-negative status was shown and CK5 was strongly expressed. (C) A graph of quantified expression levels for 10 biomarkers in the HCC70 cell line. Although the cell line was derived from the same patient and classified by luminal subtype and adeno-carcinoma with AU-565, the expression of p63 differentiated the cell lines with the same subtype.

extensively using DNA microarrays [7–9]. Proteomic profiling with the quantitative IHC/ICC method can support the extensive knowledge of breast cancers and classify the subtypes of cancers in a fast and inexpensive manner, which might a promising approach for personalized medicine [16].

We examined proteomic profiling of four breast cancer cells with the 10 biomarkers related to breast cancer via the microfluidic platform. Fig. 5A exhibits biomarkers' expressions after the ICC process for the HCC70 breast cancer cell line. Fig. 5B shows the image-analyzed result for the original immunocytochemical staining using the Gaussian mixture model and the expectation-maximization method [21]. Fig. 5C and D is the magnified images of the dotted boxes in Fig. 5A and B, respectively. Because the dimension of one biomarker was 300  $\mu$ m, a 400× microscopic view fully covered the area of cells examined via a biomarker. As shown in the images, only brown colors clearly remained, so that quantitative proteomic profiling was expected.

Fig. 6 showed quantitative proteomic profiling of breast cancer cells for 10 biomarkers. Because all biomarker expression was presented on a single cell block, the unexpected variation that may arise from multiple ICC steps was eliminated, meaning that a more credible quantitative comparison may be possible between biomarkers' expressions. Previous studies have reported that the AU-565 and SK-BR-3 cell lines were classified by luminal subtype and adenocarcinomas [9,24] and were derived from the same patient, implying that the cell lines have the same characteristics. In the profiling study, although the two cancer cell lines AU-565 (Fig. 6A) and SK-BR-3 (Fig. 6D) showed a similar expression pattern, weak expression of p63 was shown in SK-BR-3. The resulting

hypothesis was that the SK-BR-3 is nearer to a metaplastic carcinoma [25]. The HCC70 cell line has been classified as basal A subtype and a ductal carcinoma. Molecular classification revealed that the basal subtype is normally ER-, PR-, and HER2-negative ("triple-negative phenotype") and shows a more aggressive clinical behavior and poor prognosis [2,7,15]. Fig. 6B shows that the HCC70 was triple negative and the profiles were consistent with those of previous studies [26–28]. Additionally, the cell line only expressed the CK5 protein, a representative basal/myoepithelial cell marker. Although p63 was also one of the basal cell markers, the cell line did not express the biomarker. The MCF-7 cell line has been also classified by luminal subtype and metastatic lobular carcinoma. The main difference in these cells was the presence of steroid hormone receptors and the HER2-negative status. In addition, the cell line did not express the p53 protein (Fig. 6C). Four breast cancer cell lines strongly expressed Ki-67, a cellular marker for proliferation. By this platform, biomarker expression could be quantitatively compared over step-wise expressions [28,29].

We also applied the platform to human breast cancer tissue. According to a process equivalent to that of a cell block, the microfluidic massive IHC was performed as a blinded test. Fig. 7A shows the result of 10 immunohistochemical stainings on a tissue sample. As shown in the figure, the tissue sample was not damaged when applying the platform. Unlike the cell blocks, the intensity pattern of staining was not homogeneous in the tissue. Fig. 7B–G show the magnified images for ER, HER2, PR, Ki-67, E-ca and p63 biomarker regions. ER and PR were strongly expressed, and Ki-67 was slightly expressed. HER2 showed negative expression, consistent with the results of the conventional automatic IHC method



**Fig. 7.** Application of the microfluidic massive IHC platform to the tissue sample of a human breast cancer patient. (A) The patient sample showed expressions of E-ca, ER, p63, PR, and Ki-67 biomarkers, and others were not expressed (40×). (B–G) Magnified images for expressed biomarkers in Fig. 7A (400×). An tissue image stained with (B) ER, (C) HER2, (D) PR, (E) Ki-67, (F) E-ca, and (G) p63.

(data not shown). From these results, we demonstrated the applicability of the microfluidic massive IHC platform for proteomic profiling of tissue samples.

For specifically targeted therapy, many significant indicators should be discovered and examined in clinical diagnoses. For example, the mechanism for change from an ER-positive nonproliferating cell phenotype to an ER-positive proliferative cell phenotype is not fully understood with regard to which critical switches are responsible [30,31]. To define ER functionality and phenotype, and to develop a more sophisticated pathological diagnosis method for molecularly heterogeneous diseases, many more IHC-compatible markers are required and many kinds of biomarkers need to be screened. The platform described here is expected to be useful for protein regulation and signaling studies. Additionally, this platform can be used to select optimal antibodies. IHC using 1D5 or 6F11 mouse monoclonal antibodies has been the most commonly used assay to evaluate the efficacy of antihormonal therapy. However, it is controversial whether SP1

antibody is an optimal reagent; it is too sensitive, because it catches ER-positive cells that are not conventionally regarded as ER-positive [32]. It is likely to be difficult to compare the efficacy for several antibodies of the same types due to expense and tissue consumption. In this case, the microfluidic platform can facilitate efficacy studies of antibodies with tissue samples. Finally, this trial of a quantitative comparison between biomarker expressions is believed to have the potential to complement and increase the abundance of information for many kinds of cancer studies in addition to genomics.

# 4. Conclusions

We simultaneously examined 20 IHC/ICC assays on a single cell block and tissue sample. Ten biomarkers for four breast cancer cell lines were quantitatively compared, and the platform was applied to tissue specimens of breast cancer patients. Our favorable results indicate that the microfluidic massive IHC/ICC platform will be useful to confirm and complement the results of similarly scaled genomic assays, such as the Oncotype DX test (which also examines 21 factors). This will help to understand the heterogeneous and complex phenomena associated with cancer and to identify novel markers for classifying solid tumors, biomarker development for IHC, the selection of optimal biomarkers, and biological pathway studies.

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# Appendix

Figures with essential color discrimination. Figs. 1 - 6 & 7 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j. biomaterials.2010.10.040.

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