Cell chip array for microfluidic proteomics enabling rapid *in situ* assessment of intracellular protein phosphorylation

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We discuss the ability to perform fluorescent immunocytochemistry, following cell fixation, using a microfluidic array of primary, nonadherent, single CD34+ stem cells. The technique requires small cell samples and proceeds with no cell loss, making it well-suited to monitoring these rare patient-derived cells. The chip allows us to correlate live cell dynamics across arrays of individual cells with post-translational modifications of intracellular proteins, following their exposure to drug treatments. Results also show that due to the microfluidic environment, the time scale of cell fixation was significantly reduced compared to conventional methods, leading to greater confidence in the status of the protein modifications studied. © *2011 American Institute of Physics.* [doi:10.1063/1.3587095]

I. INTRODUCTION

The assessment of intracellular protein levels and post-translational modifications, e.g., protein phosphorylation, is essential for investigating cell signaling events. Conventional methods, such as Western blotting, flow cytometry, and immunofluorescence, are relatively time-consuming and their sensitivity requires large numbers of cells [see Table I in ESI (Ref. 1)]. Large numbers of cells are not always available, and often a number of sample processing steps (e.g., wash and centrifugation) result in a significant reduction in the numbers of available cells. This is an important problem when studying already rare cell populations, e.g., stem cells, where it may not be possible to obtain sufficient cell numbers to undertake conventional analyses.

The miniaturization of laboratory procedures using microfluidic technology has inherent advantages compared to conventional benchtop methods.[2] Miniaturization in a microfluidic platform allows the experimentalist to work at low Reynolds numbers (0.01 for the devices used here, for example), where viscous forces are dominant. The laminar regimes obtained under the flow conditions used, coupled with the short distances for diffusion, result in reduced time scales for reagents to reach equilibrium. This not only enables drugs and reagents to be delivered in a predictable and fast manner, but following observations on the live cell, it is also possible to bring about rapid cell fixation and immunocytochemical staining. This has the effect of accurately "freezing" the cell's proteomic status at a predetermined time, without there being continued activity.

A further advantage of microfluidic miniaturization in an array platform is the ability to conduct experiments using small total cell numbers (<1000 cells), each of which can be analyzed individually using live cell imaging.[3–6] For example, sieves have been designed to minimize the influence of liquid flow on adherent MCF7 breast cancer cells[7] and create high density microfluidic arrays to study toxity.[6,8] By decreasing the time needed for fixation and staining, microfluidics

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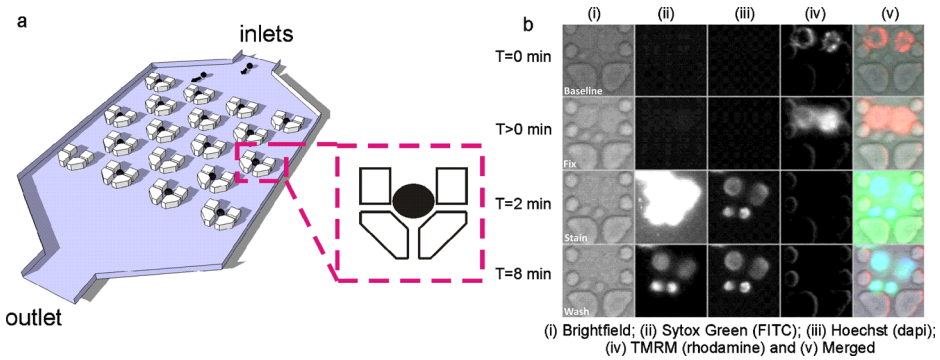

FIG. 1. Rapid cell fixation. (a) Schematics of the microfluidic cell capture. Cells and reagents are perfused through the array from the top inlets and are captured by the mechanical traps.[5] See supplementary Fig. S1 for details (Ref. 1). (b) Primary CD34+ stem cells were stained with TMRM (iv) to label mitochondria prior to loading into the microfluidic device. Cells were exposed to PBS with 20% Cytofix/Cytoperm solution. After exposing cells to fixation solution for 2 min ($T>0$ min), the device was then perfused for 2 min ($T=2$ min) with PBS containing 1 nM Sytox Green (ii) and 1 μM Hoechst dye (iii) to label dead cells and cell nuclei, respectively. Finally, cells were washed with PBS for 3 min to remove any unbound dye. The bottom row of the images shows cells stained with Sytox Green and a total loss of mitochondrial stain, indicating cell death by fixation. This image series illustrates the speed at which cells are fixed and stained within the PDMS microfluidic device.

would allow changes in intracellular protein expression/activity to be directly correlated with dynamic live cell events at the single cell level. Table I in ESI (Ref. 1) contains a comparison with conventional methods, such as Western blotting and flow cytometry, showing the potential benefits of microfluidics.

However, determining the advantage of using a microfluidic device is, in itself, challenging, as it is generally unknown how quickly cells become fixed using conventional methods. Laboratory-based protocols require a minimum of 10 min for cell fixation[9] and it is likely that during these extended periods of time, some intracellular signaling pathways may proceed. Thus, the more rapidly intracellular proteins become cross-linked (i.e., "frozen" or fixed), the more accurate the information obtained regarding the signaling state of the cell is likely to be at any time point. This is of particular importance when investigating rapid intracellular signaling dynamics, e.g., protein phosphorylation status, in response to drugs or changes in the cell's microenvironment.[10]

Previously, we have demonstrated the ability to monitor live cell dynamics in individual CD34+ hematopoietic stem/progenitor cells[5] from patients with chronic myeloid leukemia (CML) and healthy controls. In this rare, primitive cell population, it is important to be able to correlate live cell events (e.g., apoptosis or cell division) with specific intracellular protein changes (e.g., activation of signal transduction pathways) in arrays of single cells. By comparing both normal and leukemic (patient-derived) cells, it may in future be possible to assess the effects of drug treatment, including small molecule inhibitors.[11]

For example, in the case of the chemotherapeutic drug dasatinib (Sprycel, Bristol-Myers Squibb), which is a multitargeted kinase inhibitor, it may be possible to understand its impact on signaling events in leukemic stem cells at the single cell level. It is already well established in the treatment of CML[12] that dasatinib specifically inhibits BCR-ABL tyrosine kinase activity and SRC family kinase activity. However, like other tyrosine kinase inhibitors, dasatinib fails to eliminate the quiescent CML stem cell fraction, postulated to result in disease persistence and, in some cases, relapse.[13] We believe that in future, our platform will enable an improved understanding of the intracellular events, which lead to insensitivity or resistance of these stem cells to drugs.

In this paper, we demonstrate the ability to rapidly fix single CML CD34+ hematopoietic stem/progenitor cells within a single cell microfluidic array [Fig. 1(a)]. In addition, we identified intracellular proteins and phosphorylated proteins in the trapped cells using conjugated fluorescent antibodies. This is, to the best of our knowledge, the first demonstration of a technique allowing to follow the same single cell through live imaging, to the investigation of intracellular protein

phosphorylation. In doing so, we describe the potential of the device in monitoring rare, nonadherent patient-derived cells, and importantly, demonstrating the ability to correlate live cell events (measured fluorescently on-chip) with immunocytochemical identification of cell signaling pathways. To illustrate the application of this technology, we explored the impact of dasatinib on the phosphorylation status of SRC and STAT5 proteins in primary CD34+ hematopoietic stem cells from CML patients.

II. MATERIALS AND METHODS

A. Cell culture

Normal and CML stem/progenitor cells were isolated as described previously.[14] Briefly, fresh leukapheresis or peripheral blood samples were obtained with written informed consent and approval of the Local Ethics Committees from patients with newly diagnosed CML and normal donors of peripheral blood stem cells. Samples were enriched for CD34+ cells using CliniMACS (Miltenyi Biotech Ltd.) according to the manufacturer's instructions and cryopreserved in 10% (v/v) dimethylsulfoxide in [5% (w/v) Human Albumin Solution, Baxter Healthcare Ltd.).

CD34+ cells were maintained in serum free medium consisting of Iscove's Modified Dulbeccos Medium (Invitrogen) supplemented with serum substitute [bovine serum albumin (BSA), insulin, transferrin (BIT; Stem Cell Technologies)], L-glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), and 0.1 μM 2-mercaptoethanol (Sigma-Aldrich). In addition, the medium was supplemented with five growth factors [20 ng/ml interleukin [IL]-3, 20 ng/ml IL-6, 100 ng/ml stem cell factor, 100 ng/ml Flt-3 ligand (all from Stem Cell Technologies), and 20 ng/ml granulocyte-colony stimulating factor (Chugai Pharma)]. Cells were maintained in a humidified 37 °C incubator with 5% CO_2. Dasatinib was kindly provided by Bristol-Myers Squibb.

B. Microfluidic array preparation

Microfluidic device design was described in detail previously.[5,14] Briefly, SU-8 masters were used to cast polydimethyl siloxane (PDMS) (Dow Corning) devices, which were irreversibly bonded to glass cover slips using plasma oxidation. Prior to use, devices were sterilized by immersion in a sonic bath containing methanol for 5 min and were then flushed with sterile cell culture media. Two trap designs were used [Figs. 1(b) and 2] enabling the trapping of either two isolated cells or single cells, respectively. Double cell traps demonstrated not only the synchronous behavior in response to immunostaining of similar cells but also the different surface and intracellular immunostaining properties of different cell types (e.g., viable or nonviable) irrespective of the local geometry within the microtrap. In addition, the double cell traps would allow for limited assessment of intercellular interactions.

First, cells were centrifuged in an Eppendorf tube for 1 min at 5000 rpm. Cells were then aspirated into the poly-ether-ether-ketone tubing (VICI International) directly from the centrifugation pellet using glass syringes (Hamilton) and microsyringe pumps (Havard Apparatus). Unless otherwise stated, flow rates between 50 and 80 nl/min (corresponding to linear velocities of approximately 4–6 mm/s) were used to load and perfuse cells.

C. Cell fixation and staining on PDMS device

Cells were fixed using a 20% (v/v) solution of Cytofix/Cytoperm solution from Cytofix/Cytoperm™ kit (Becton Dickinson) for the time specified for each individual experiment. Solutions containing 1 nM Sytox Green (Invitrogen), 2.5 μg/ml Annexin-V-PE or Annexin-V-Cy5 (BD Biosciences), 1 μM Hoechst (Invitrogen), 25 μl AlexaFluor 488 conjugated phosphorylated SRC (p-SRC; Tyr416) antibody (Upstate Biotechnology), and/or 25 μl AlexaFluor 488 conjugated phosphorylated STAT5 (p-STAT5; Tyr694) antibody (BDTM Phosflow, BD Biosciences) were used as mentioned throughout the text. Images were collected using a standard 20× NA 0.75 Fluar objective with an inverted AxioObserver Z1 microscope. A mounted temperature controlled chamber and microfluidic pumps enabled cell array perfusions at 37 °C.

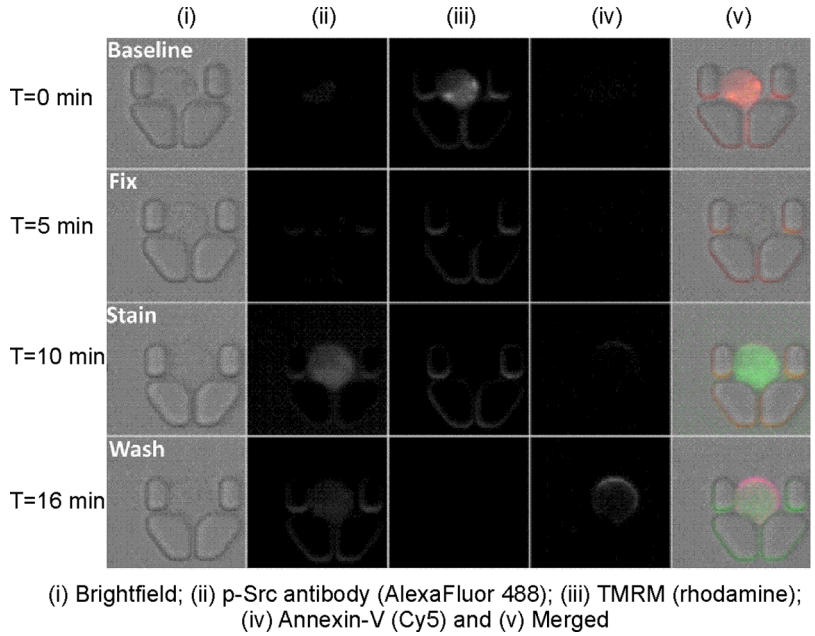

FIG. 2. Intracellular immunocytochemistry following fixation. (a) Primary CML CD34+ stem/progenitor cells were labeled with TMRM mitochondrial stain (iii) prior to loading into the PDMS microfluidic device and then perfused with media containing Alexa-Fluor 488 conjugated p-SRC antibody (Tyr416) (ii) and Annexin-V-Cy5 (iv), which do not stain the cells at first, showing that they are viable and not permeated (T=0 min). Once cells were exposed to the fixation solution (T=5 min), the TMRM dye dissipated nearly instantaneously. After allowing cells to fix for 2 min, the flow was switched back to the initial solution containing p-SRC antibody and Annexin-V for 3 min to stain the cells (T=10 min). Finally, the cells were washed with PBS for 5 min. The bottom row (T=16 min) shows a cell stained for p-SRC and Annexin-V, indicating that it is both dead (by fixation) and labeled with an intracellular antibody (p-SRC).

Fluorescent images were analyzed using the AXIOVISION software (Zeiss). Regions of interest delimiting the cells in the traps were outlined manually and the fluorescence inside these was quantified. Cells were counted as stained when the fluorescent signal exceeded a cut-off established 3 standard deviations above the mean of the signal for unstained cells. A total of at least 200 cells was analyzed for each condition. Student t-tests were used to evaluate the statistical significance of the results.

D. Flow cytometry

At the end of the stated culture periods, CD34+ stem/progenitor cells were labeled with Annexin-V-PE to assess apoptosis. Apoptotic cells were defined as Annexin V-PE+. Intracellular p-Src and p-STAT5 staining were performed and analyzed by flow cytometry using methods described previously.[13] Briefly, to assess p-SRC or p-STAT5, at least 1×10^5 CD34+ CML cells were resuspended in 100 μl fixing reagent from a FIX&PERM® kit (Caltag Laboratories) and incubated at room temperature for 15 min. The cells were then washed with 3 ml phosphate buffered saline (PBS)/0.1% BSA/0.1% azide buffer (PBS/BSA/azide) and centrifuged at 120*g* for 5 min. The supernatant was poured off and 50 μl permeabilizing reagent was added. 1 μl of p-SRC antibody, 10 μl p-STAT5, or 2 μl IgG control was added directly to this buffer, the cells were vortexed, and then incubated at room temperature for 40 min. The wash step was then repeated twice and the cells were resuspended in 100 μl PBS/BSA/azide. All fluorescence-activated cell sorter or sorting analysis was performed using a FACSCanto (BD Biosciences).

III. RESULTS AND DISCUSSION

A. Rapid *in situ* fixation of primitive hematopoietic cells in microarrays

The dynamic process of cell fixation using our microfluidic platform is shown in Fig. 1(b). In this configuration, live CD34+ stem/progenitor cells were preloaded with the intracellular mitochondrial stain, tetramethylrhodamineester (TMRM) (Invitrogen) and perfused with serum free medium containing 5 growth factors[5] and 1 nM Sytox Green to label dead cells. In a similar fashion as our previous work,[5] this illustrates that live cell dynamics information can be obtained on single cells registered in the array, viability and mitochondria location and quantities, for example. These cells were then exposed to a fixation solution containing 20% v/v Cytofix/Cytoperm (Becton Dickenson), which resulted in the depolarization of mitochondria and dispersal of the TMRM dye out of the cells, in less than 2 min. In Fig. 1, we chose a trap geometry providing space for two cells to illustrate the synchronicity of events within the platform. The speed at which the mitochondria were affected by the introduction of fixation solution indicated that the intracellular dynamics were instantaneously affected. This is particularly important when interrogating intracellular pathways, as delay in fixation, resulting from less well controlled mass transfer, could result in protein expression pathways continuing to develop and possibly lead to an ambiguous readout.

Following cell fixation, we then proceeded with intracellular immunostaining using fluorescent dyes. The cells were perfused for 2 min with PBS containing both 1 nM Sytox Green (to label dead cells) and 1 μM Hoechst (to label the cell's nuclei). The cells in the device were then washed with PBS to rinse away unbound dye. The concomitant total loss of the intracellular mitochondrial dye and signal from Sytox Green fluorescence show that the cells are dead. As shown in Fig. 1(b), the entire process of cell fixation, staining, and washing required only 8 min, compared to a minimum of 80 min for intracellular flow cytometry and more than 1 day for Western blotting [Table I of ESI (Ref. 1)]. More importantly, looking at rare, stem cell populations, the process results in little cell loss after the cells are trapped. The technique also allows for many cells to be examined in parallel and it appears to be relatively benign regarding cellular integrity (with less than 10% showing damage). This may be important if probing for less stable proteins (e.g., BCR-ABL) or membrane bound complexes.[15]

B. Rapid *in situ* immunostaining for intracellular phosphorylated proteins in microarrays

Having established that cell fixation proceeded rapidly in a microfluidic environment, we then determined if, following fixation, intracellular immunostaining of cells using conjugated fluorescent antibodies was possible, in a similarly rapid fashion (Fig. 2). Again, cells were prelabeled with the mitochondrial dye TMRM prior to loading into the microfluidic device. As previously, once the cells had been exposed to the perfusate containing the fixative solution, loss of TMRM staining became apparent. Cells were then perfused for 10 min with a solution of PBS containing Alexa-Fluor 488 conjugated p-SRC antibody as well as Annexin-V-Cy5 and were then washed with PBS. As shown at the bottom panel of Fig. 2, even with a short incubation period of 3 min with the labeled antibody, the cells were clearly stained, indicating successful cell fixation with both surface (Annexin-V) and intracellular (p-SRC) protein staining with fluorescent antibodies.

C. Inhibition of p-SRC and p-STAT5 in individual CD34+ CML cells in response to dasatinib

To confirm the specificity of the antibody labeling procedure, we investigated the expression of p-SRC and p-STAT5 before and after exposure to dasatinib in CD34+ CML cells. The multitargeted kinase inhibitor dasatinib specifically inhibits the tyrosine phosphorylation of BCR-ABL in CML, resulting in the inhibition of BCR-ABL function with suppression of downstream effector pathways, such as JAK-STAT, SRC, PI3 kinase, and RAS.

Figure 3 shows that CD34+ CML cells at baseline, prior to dasatinib stained positively for p-SRC and p-STAT5 (the activated forms of these proteins), whereas cells fixed following 24 h

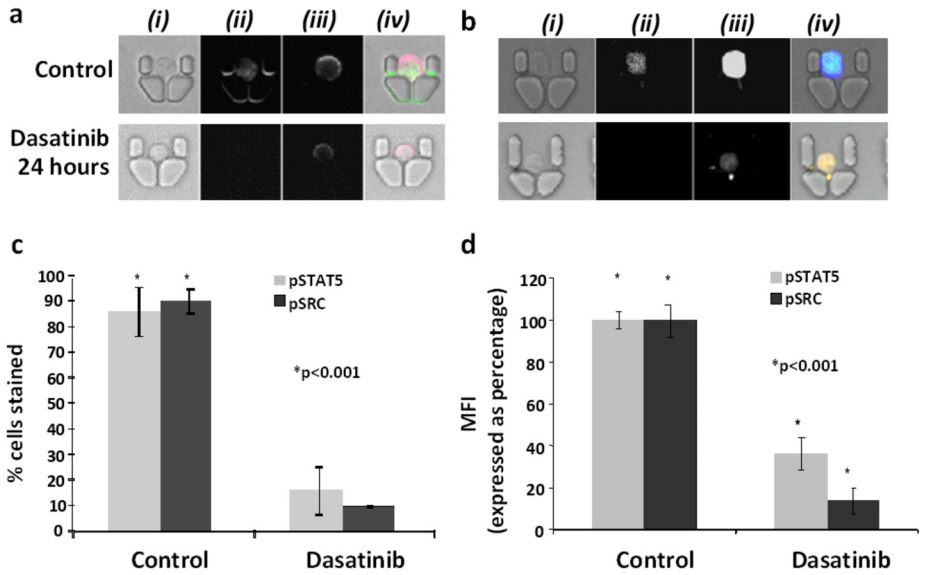

FIG. 3. Patient-derived CD34+ stem cells fixed and stained for p-Src (Tyr416) and p-STAT5 (Tyr694) presence in no drug control and dasatinib-exposed cohorts. (a) Cells labeled with antibodies for p-SRC, where panels represent (i) bright field, (ii) Alexa-Fluor 488-conjugated p-SRC antibody, (iii) Annexin-V-Cy5, and (iv) merged images. (b) Cells labeled with antibodies for p-STAT5, where panels represent (i) bright field, (ii) Alexa-Fluor 488 conjugated p-STAT5, (iii) Sytox green, and (iv) merged images. Note that in both (a) and (b), control populations stain positively with p-SRC or p-STAT5, while populations exposed to dasatinib for 24 h do not. These results are quantified in graph (c), showing the percentage of the cells stained in control and dasatinib-exposed populations. Clearly, the inhibition of tyrosine kinase activity by dasatinib is reflected in the absence of phosphorylated proteins within the cells. (d) The results are further validated in parallel flow cytometry experiments that confirm the inhibition of p-SRC and p-STAT5 in response to dasatinib after 24 h of exposure to drug. MFI stands for mean fluorescence intensity.

exposure to dasatinib exhibited no staining. While only single cell traps are presented in the images, a quantitation of all cells trapped, shown in Fig. 3(c), indicated the same trend.

These results were then benchmarked in a parallel flow cytometry study, which confirmed the inhibition of p-SRC and p-STAT5 by dasatinib [Fig. 3(d)]. While flow cytometry required the analysis of more than 10^4 cells and the preparation of 10^5 cells, the microfluidic device was loaded with only a few hundred cells. These results show the capability of the microfluidic method to investigate rare cell populations that would not be available in sufficient quantities for flow cytometry, such as patient-derived, nonadherent hematopoietic stem cells (HSCs).

Moreover, the bulk sample processing for flow cytometry (involving centrifugation, for example) precludes the correlation between dynamic results, obtained on the cells in culture, and the protein expression results, obtained after fixation. This is in contrast to the results for the cell array, where dynamic changes measured by live cell fluorescence imaging could then be correlated with immunostaining following cell fixation. This advantage is even more pertinent in the case of Western blotting (rather than for cytometry), see Table I in ESI,[1] as the blotting technique relates an average protein expression level for all the cells analyzed. In contrast, the single cell array allows both dynamic and intracellular expression study on the same cell.

IV. CONCLUSION

We have shown the application of microfluidic single cell arrays to investigate intracellular protein post-translational modifications, by immunostaining following cell fixation *in situ*. The technique requires only a few hundred cells and proceeds without significant sample loss. It is thus well-suited for monitoring rare cell populations from patients, such as nonadherent primary cells, normal, and leukemic HSC, which may not be available in sufficient quantities to be analyzed through conventional techniques, and which are difficult to monitor using conventional wide field

microscopy. The advantage of using this microfluidic platform compared with flow cytometry, or other benchtop techniques that may approach single cell resolution, is our ability to correlate the live cell dynamics to the intracellular protein expression patterns for each individual cell. We also show for the future that the microfluidic environment results in faster time scales for the processes of fixation and staining, in effect stopping the protein activity at a specific time point, which increases the confidence in the relationships studied.

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[1] See supplementary material at http://dx.doi.org/10.1063/1.3587095 for Table I showing the comparison with conventional techniques and Fig. S1 showing experimental setup schematics.
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