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# Patterning pallet arrays for cell selection based on high-resolution measurements of fluorescent biosensors

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ABSTRACT

Pallet arrays enable cells to be separated while they remain adherent to a surface and provide a much greater range of cell selection criteria relative to that of current technologies. However there remains a need to further broaden cell selection criteria to include dynamic intracellular signaling events. To demonstrate the feasibility of measuring cellular protein behavior on the arrays using high resolution microscopy, the surfaces of individual pallets were modified to minimize the impact of scattered light at the pallet edges. The surfaces of the three-dimensional pallets on an array were patterned with a coating such as fibronectin using a customized stamping tool. Micropatterns of varying shape and size were printed in designated regions on the pallets in single or multiple steps to demonstrate the reliability and precision of patterning molecules on the pallet surface. Use of a fibronectin matrix stamped at the center of each pallet permitted the localization of H1299 and mouse embryonic fibroblast (MEF) cells to the pallet centers and away from the edges. Compared to pallet arrays with fibronectin coating the entire top surface, arrays with a central fibronectin pattern increased the percentage of cells localized to the pallet center by 3–4-fold. Localization of cells to the pallet center also enabled the physical separation of cells from optical artifacts created by the rough pallet side walls. To demonstrate the measurement of dynamic intracellular signaling on the arrays, fluorescence measurements of high spatial resolution were performed using a RhoA GTPase biosensor. This biosensor utilized fluorescence resonance energy transfer (FRET) between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to measure localized RhoA activity in cellular ruffles at the cell periphery. These results demonstrated the ability to perform spatially resolved measurements of fluorescence-based sensors on the pallet arrays. Thus, the patterned pallet arrays should enable novel cell separations in which cell selection is based on complex cellular signaling properties.



## 1. Introduction

There is currently great interest in studying the mechanisms of spatially isolated behaviors in cells such as calcium sparks and waves, pseudopodia extension and retraction, invadopodia dynamics, focal adhesion formation, endoplasmic reticulum assembly and disassembly among many others. A plethora of probes have now been developed to track this localized signaling behavior for example fluorescent monitors for calcium and other ions, fluorescence resonance energy transfer (FRET)-based indicators for kinase proteases and GTPases, translocation probes to monitor protein domain movement, fluorescent highlighters to identify protein complex age and assembly/disassembly, and small metabolite sensors [1–3]. Biosensor design and synthesis has been particularly successful for kinases [4] which add a phosphoryl to a protein or lipid and GTPases such as the Rho family [5] which cycle between active and inactive states in a nucleotide-dependent manner. Live cell imaging studies have revealed the subcellular spatial and temporal dynamics of activation for these enzymes in single cells. Use of the biosensors has also revealed the diversity of signaling among single cells within a population. While these measurements have been performed at the single-cell level, cells identified as having interesting signaling variants have not been obtainable as a pure population and are thus unavailable for future studies. This is largely due to the spatially and temporally dynamic behavior of cellular signaling enzymes and the absence of cell separation technologies compatible with cell selection based on high resolution measurements in both time and space. Methods such as fluorescence-activated cell sorting (FACS) or magnetic-activated

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cell sorting (MACS) offer no or extremely limited spatial and temporal measurements of biosensor behavior in cells. However, much utility lies in the use of live cell biosensors for the screening and isolation of cells displaying unique signaling behaviors in response to altered environmental conditions, drug applications, or other perturbations.

Pertz et al. have previously reported an intra-molecular FRET biosensor for the small GTPase Rho [6]. Upon RhoA activation by GTP-loading, RhoA interacts with a tethered Rho-binding domain (RBD), altering the relative orientation of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) within the tether. In migrating mouse embryonic fibroblasts (MEFs), RhoA activation is present in a tight band of activity at the leading edge of the cell as well as transiently during tail retraction [6]. With this biosensor and others, the spatial and temporal relationship between RhoA activity and the GTPases Rac1 and Cdc42 was elucidated, showing that RhoA is activated first among these GTPases at the cell edge, followed by Rac1 and Cdc42 activation occurring at a zone away from the edge with a ~40 s time delay [7]. Genetic or pharmacological perturbation of these spatial or temporal activation signatures followed by selection and separation of cells with unique molecular activation states would be of high utility in investigations of RhoA signaling.

Microarrays varied in type, size, and function have been a part of the biomedical experimentalists' tool box for many years [8,9]. Arrays of releasable elements or pallets which are made of photoresists such as SU8 and 1002F were recently introduced for the separation of adherent cells [10–13]. Cells arrayed on the releasable elements can be screened by conventional microscopy-based assays and instrumentation. Selected cells can then be released from the underlying substrate, collected and expanded [9]. Despite the advantages that these releasable microarrays provide in screening and separating cells [8–13], the technology possesses limited utility for separations based on fluorescence measurements of high spatial resolution, for example, the measurement of subcellular signaling events. This constraint is primarily due to intense light scattering at the pallet edges which interferes with fluorescence measurements in adjacent cellular regions as well as to the very short working distance of the required high-numerical aperture (NA), oil-based objectives. The origin of the light scatter is the differing refractive indices between the pallet and surrounding medium. Light scattering from the pallet edges into the microscope objective during imaging is likely enhanced by the rough pallet side walls. Further, the greater roughness at the junction of the pallet surface and side wall results in a preference for cell attachment near the edges of the pallet where scattering is at its greatest intensity. The small working distance of the high NA objective also necessitates an array of minimum thickness. Thus the acquisition of high resolution fluorescence images of cells on the pallets to screen and select cells based on biosensor readouts of subcellular processes has not been possible.

The goal of this work was to demonstrate the feasibility of high resolution imaging of RhoA activity in cells on the pallet arrays. To achieve this goal and minimize optical artifacts, the pallet surfaces were patterned to direct cell attachment to the center of the pallet and spatially separate cellular fluorescence from the scattered light. The novelty of this work lies in the successful adaptation of the arrayed releasable elements or pallets so that the elements were compatible with high resolution imaging of fluorescent biosensors over time. This includes the adaptation of the array for the high NA objectives as well as surface patterning to spatially segregate light scatter. These modifications enable highly sensitive, temporally, and spatially resolved fluorescence measurements of cells cultured on the pallet arrays which has the potential to enable cell selection and separation based on complex, dynamic protein behavior, a feat not currently possible. This achievement would greatly broaden the type of selection criteria used to separate a mixed population of cells potentially identifying cells with variant pathways or unique drug responses.

## 2. Experimental

### 2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, puromycin, doxycycline, Ham's F-12K medium without phenol red, phosphate buffered saline (PBS) and 0.05% trypsin with EDTA were obtained from Invitrogen (Carlsbad, CA). HEPES buffer was purchased from Mediatech, Inc. (Manassas, VA). EPON resin 1002F (phenol, 4,4′-(1-methylethylidene)bis, polymer with 2,2′-[(1-methylethylidene)bis(4,1-phenyleneoxymethylene]bis[oxirane])) was acquired from Miller-Stephenson (Sylmar, CA). SU8 photoresist and SU8 developer (1-methoxy-2-propyl acetate, also used for 1002F) were from MicroChem Corp. (Newton, MA). Heptadecafluoro-1,1,2,2-tetrahydrodecyl trichlorosilane was from Gelest Inc. (Morrisville, PA). Human plasma fibronectin was purchased from Millipore Corporation (Billerica, MA). Unless otherwise said in the text, all other chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

### 2.2. Fabrication of pallet arrays

Arrays of 1002F (130 μm sides, 50 μm spacing, and 50 μm height unless stated otherwise) were fabricated on glass cover slips (Warner Instruments, Hamden, CT) as described previously [14]. The use of number one cover slips (thickness ~150 μm) was necessary in order to perform cell fluorescence measurements with high numerical aperture objectives. Chambers (25 mm size) surrounding the arrays were constructed from poly(dimethyl siloxane) (PDMS) using a Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) as described previously [10]. To eliminate cell attachment between the pallets, virtual air walls were created between the pallets using a vapor-phase deposition with heptadecafluoro-1,1,2,2-tetrahydrodecyl trichlorosilane overnight as described previously [8]. After an overnight coating, the arrays were kept on a 120 °C hot plate for 2 h and the remaining chemical residue washed with ethanol and the array dried with a $N_2$ stream. To enhance adhesion of matrices to the pallet, the pallet surfaces were roughened for 30 s as described previously [13]. The arrays were then washed with water and ethanol and dried in stream of $N_2$. The arrays were stored in a vacuum desiccator until use.

### 2.3. Fabrication of PDMS stamps

1002F molds in various shapes and sizes (see Section 3) for forming the PDMS stamps were fabricated and coated overnight by vapor-phase deposition with heptadecafluoro-1,1,2,2-tetrahydrodecyl trichlorosilane as described above. A 1:8 (catalyst:monomer) PDMS mixture was layered over the 1002F mold. In order to prevent shrinking or expansion of the PDMS stamp after release from the mold, the uncured PDMS mixture was covered with a 50-μm thick 1002F film fabricated on 1-mm thick microscope glass slide. The assembly was then degassed for 1 h and cured for 1 h at 65 °C. After slowly cooling to room temperature, the PDMS stamp was carefully cut and peeled from the 1002F mold while it remained bonded to the underlying support of 1002F film on glass. The PDMS stamp was roughened for 25 s as described previously [13]. After gently washing with water and ethanol followed by drying under an $N_2$ stream, the PDMS stamps were placed in an air-plasma cleaner (Harrick PDC-001, Ithaca, NY) for 20 min immediately before use.