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## Single cell patterning for high throughput sub-cellular toxicity assay

Junfei Xia<sup>a</sup>, Yuting Qiu<sup>a</sup>, Xiaojie Xun<sup>a</sup>, Liyuan Ma<sup>a</sup>, Jingjiao Guan<sup>b</sup>, Ming Su<sup>a,\*</sup>

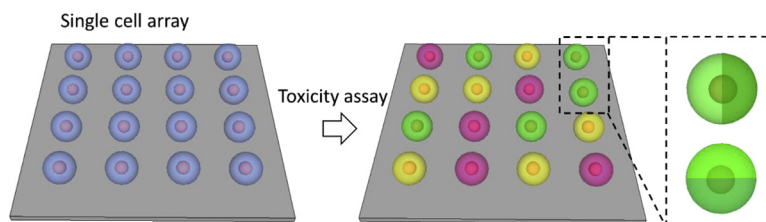
<sup>a</sup> Department of Chemical Engineering, Northeastern University, Boston, MA 02115, USA

<sup>b</sup> Department of Chemical and Biomedical Engineering, Florida State University, Tallahassee, FL 32310, USA

### HIGHLIGHTS

- Single cell array is made by selective cell adhesion onto lithographically patterned surface.
- Population based toxicity, single cell toxicity, and subcellular toxicity are derived simultaneously at high throughput.
- Issues of cell overlapping and clustering are completely solved.
- Imaging software allowed for rapid, objective and automated extraction of toxicity information without user interference.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Cell population based toxicity assays cannot distinguish responses of single cells and sub-cellular organelles; while single cell assays are limited by low statistical power due to small number of cells examined. This article reports a new single cell array based toxicity assay, in which cell responses at population level, single cell level and sub-cellular level can be obtained simultaneously at high throughput. The single cell array was produced by microcontact printing and selected area cell attachment, and exposed to damaging X-ray radiation, which was followed by fluorescence imaging after staining. Two image processing softwares written in Python and MATLAB were used to determine the expressions of proteins associated with cell migration and invasion, and production of reactive oxygen species (ROS), respectively. The results showed significant differences in responses at single cell level and distinctive molecular heterogeneity at sub-cellular level in a large population of cells upon exposure to radiation.

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### 1. Introduction

A panel of in vitro toxicity assays has been developed to assess the effects of chemicals and external stimuli on cultured mammalian cells for drug screening and hazard identification [1–4]. The toxicity assays depend on cell functions such as enzyme

activity, membrane permeability, adherence, adenosine triphosphate (ATP) and co-enzyme productions, and nucleotide uptake activity [5–7]. The assays can provide averaged signals from a large population of cells. However, due to cellular heterogeneity, the toxicity results derived from cell population can be irrelevant or misleading especially in the case of cancer which is known for high level of heterogeneity [8,9]. Furthermore, toxicity assays relying on non-image based readouts cannot reveal sub-cellular distribution of events and cannot help to understand the mechanism of responses. Fluorescence imaging allows mapping of spatial

\* Corresponding author.

E-mail address: [m.su@northeastern.edu](mailto:m.su@northeastern.edu) (M. Su).

distribution of cellular events with specific dyes, but is limited by cell overlapping issue and a small number of cells that can be counted under a microscope.

Single cell toxicity assay can distinguish the function and behavior of individual cells from a background of million cells, and identify subpopulations in a heterogeneous mixture of cells or cell sub-types [10–12]. Single cell toxicity assays are often performed in dynamic mode with flow cytometry or microfluidics, which has high linear speed, but does not provide information on cell-cell communication, cell morphology and sub-cellular features [13–17]. An ideal single cell toxicity assay should be able to provide cell information on three different levels: cell population, individual cells, and sub-cellular level, which are required for proper extrapolation of in vitro toxicity response to in vivo response [18,19]. In this perspective, an ordered array of cells attached on a planar substrate is a better choice by allowing observation of cell population, individual cells, and sub-cellular organelles [20–25]. Single cell array as a platform technology has been used for many biology-driven applications such as single cell fluorescence in situ hybridization (FISH), genotoxicity study, and RNA sequencing [26–28]. A variety of methods can be used to generate single cell arrays on a solid substrate after modifying the substrate with positively charged molecules, cell adhesive ligands, and specific antibodies [26,29–32]. By attaching cells onto the substrate, it is also feasible to conduct time-dependent analysis of cell population [23].

This article describes a single cell array based toxicity assay, in which population based toxicity, single cell toxicity, and sub-cellular toxicity can be derived simultaneously and at high throughput (Fig. 1). The single cell array is made by attracting onto micropatches produced with microcontact printing of polyelectrolyte multilayers. Cell responses to ionizing radiation were simultaneously obtained at three distinct levels. By physically attaching cells at the same height and ordered locations, the issues of cell overlapping and clustering associated with random distribution were solved completely, and there is no need for a user to change observation field to find cells that can provide useful information. The image processing softwares developed in this work allowed rapid, objective and automate extraction of toxicity information without user interference. By uniquely providing population based assay with sub-cellular spatial resolution and single cell sensitivity, this method has the potential to significantly impact the toxicity field.

## 2. Materials and methods

Poly(allylamine hydrochloride) (PAH) with molecular weight of 120,000–200,000 was from Alfa Aesar. Poly(sodium 4-styrene sulfonate) (PSS) with molecular weight of 70,000, fluorescein isothiocyanate isomer I (FITC), monoclonal anti-vinculin-FITC antibody, and anti- $\beta$ -tubulin antibody were from Sigma. Hydroxy(polyethyleneoxy) propyl triethoxysilane (PEG-silane) (8–12 ethylene oxide, 50% in ethanol) was from Gelest. Polydimethylsiloxane (PDMS Sylgard 184) was from Dow Corning. Goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor 568, Hoechst 33342, and Carboxy-H2DCFDA were from ThermoFisher. Polyelectrolyte stock solutions were 1 wt% PAH at pH 10, 1 wt% PSS at pH 5.8, and 1% PAH at pH 5.8, all contain 150 mM NaCl.

A mixture of PDMS prepolymer and curing agent (10:1 wt ratio) was poured onto a master prepared by photolithography. After being kept at 37 °C for 24 h, the solidified PDMS slab was peeled off and cut into square stamps that have vertical micro-pillars with a diameter of 10  $\mu$ m, a height of 5  $\mu$ m, and a center-to-center distance of 30  $\mu$ m in the hexagonal lattice. A glass slide was treated with high level oxygen plasma for 3 min, and soaked in PEG-silane solution (5% in ethanol) for overnight, and rinsed with water and dried with an air stream.

Single cell array is formed as follows. A  $1 \times 1$  cm<sup>2</sup> PDMS stamp with micropillars was immersed in positive and negative polyelectrolyte solutions repeatedly for 15 min each and rinsed with water to form multilayers. After drying in air, the stamp was exposed to vapor from a water bath, and immediately brought into contact with a PEG-coated slide for 20 min to allow water between stamp and slide to evaporate, which is followed by peeling off the stamp to complete the pattern transfer. To enclose cell suspension onto the patterned substrate, a PDMS slab with a 5 mm diameter hole was placed on the pattern to form a chamber, into which 200  $\mu$ L cell suspension ( $5 \times 10^5$  cell/mL) in culture medium was added. The cells in chamber were incubated at 37 °C and 5% CO<sub>2</sub> for 1 h, followed by removing PDMS slab and washing the cell adhesion area gently with phosphate buffer saline (PBS) to remove unbound cells. The cells were immersed in PBS during the assay process.

CEM cells were cultured in RPMI 1640 medium supplemented with 10% (volume) fetal bovine serum (FBS), 100 units/mL of penicillin, and 100  $\mu$ g/mL streptomycin at 37 °C and 5% CO<sub>2</sub>. HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin at 37 °C and 5% CO<sub>2</sub>.

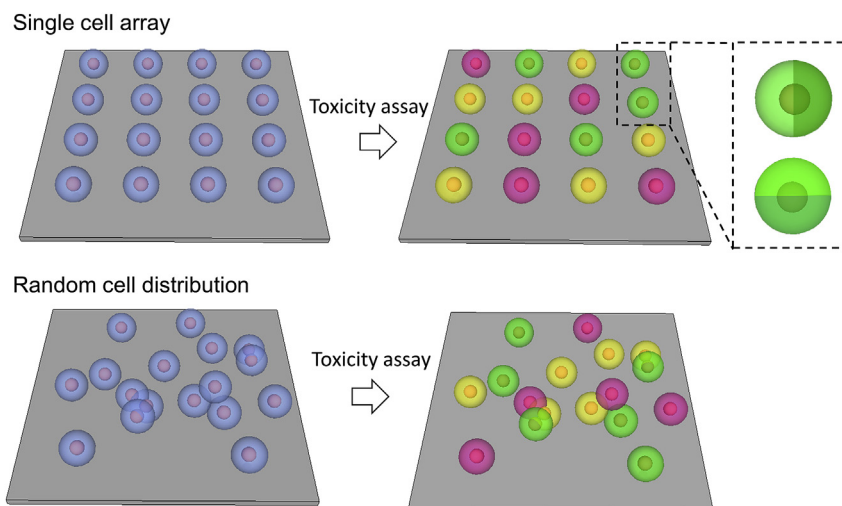


Fig. 1. Single cell patterning for high throughput sub-cellular toxicity assay.

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