***In situ*, dual-mode monitoring of organ-on-a-chip with smartphone-based fluorescence microscope**

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ABSTRACT

The use of organ-on-a-chip (OOC) platforms enables improved simulation of the human kidney's response to nephrotoxic drugs. The standard method of analyzing nephrotoxicity from existing OOC has majorly consisted of invasively collecting samples (cells, lysates, media, etc.) from OOC. Such disruptive analyses potentiate contamination, disrupt the replicated *in vivo* environment, and require expertise to execute. Moreover, traditional analyses, including immunofluorescence microscopy, immunoblot, and microplate immunoassay are essentially not *in situ* and require substantial time, resources, and cost. In the present work, the incorporation of fluorescence nanoparticle immunocapture/immunoagglutination assay into an OOC enabled dual-mode monitoring of drug-induced nephrotoxicity *in situ*. A smartphone-based fluorescence microscope was fabricated as a handheld *in situ* monitoring device attached to an OOC. Both the presence of γ -glutamyl transpeptidase (GGT) on the apical brush-border membrane of 786-O proximal tubule cells within the OOC surface, and the release of GGT to the outflow of the OOC were evaluated with the fluorescence scatter detection of captured and immunoagglutinated anti-GGT conjugated nanoparticles. This dual-mode assay method provides a novel groundbreaking tool to enable the internal and external *in situ* monitoring of the OOC, which may be integrated into any existing OOCs to facilitate their subsequent analyses.

Key words: Particle immunoagglutination; Kidney-on-a-chip; γ -glutamyl transpeptidase; 786-O cells; Smartphone microscope.

1. INTRODUCTION

Organ-on-a-chip (OOC) is defined as an integration of advanced 3D tissue engineered constructs with microfluidic network systems, i.e., lab-on-a-chip (LOC) (Bhise et al., 2014). Human cells are seeded and proliferated within the LOCs, providing a realistic replicate of the human organ. Such novel platforms improve preclinical testing of drugs, implants, biomedical devices, or stem cell therapies, due to their more realistic simulation of physiological function. OOCs have been developed to mimic the functions of various organs, including kidney, liver, brain, heart, skeletal muscle, and intestine (Ghaemmaghami et al., 2012; Esch et al., 2011). In OOCs, fluid flow generates mechanical forces that recapitulate the *in vivo* microenvironment experienced by cells, which cannot be accomplished by static 2D mammalian cell culture (Bhise et al., 2014). In this work, we are interested in developing kidney-on-a-chip to assess chemical-induced toxicity. Kidney is the main excretory organ that is exposed to drugs and xenobiotics. The epithelial cells of the renal proximal tubules in kidney are the most susceptible target for such drugs and xenobiotics (referred to as nephrotoxics), due to their roles in the concentration of glomerular filtrate and their capacity for drug metabolism (Tiong et al., 2014). Specifically, previous studies of kidney-on-a-chip observed that proximal tubular cell (PTCs) functionality and morphology are improved under flow conditions (Jang et al., 2013), which is critical to emulate a response similar to the human kidney.

However, assessment of cell response during such OOC experiments remain tedious, disruptive, time-consuming, and lack real-time *in situ* analyses (Cai et al., 2015; Jang et al., 2013; Johnson et al., 2016; Maschmeyer et al., 2015). For example, a reported kidney-on-a-chip required immunofluorescence microscopy of the membrane within the OOC via cell fixation to monitor the growth of the epithelial cells (thus not *in situ*) and immunoblot of cells harvested from the OOC post-treatment (Jang et al., 2013). Moreover, both methods require overnight

analyses steps and invasive collection of the membrane and cells. Consequentially, the irreversible, permanent bonding of polydimethyl siloxane (PDMS)-based OOCs, the most common material used for not only OOCs but also bulk of LOCs, challenges the users in accessing, isolating, and processing cultured cells for certain preferred analysis including histology and electron microscopy (Huh et al., 2013). Thus, the users are subject to destructive methods of subsequent analyses of cells, or may heavily rely on fluorescence microscopy after fixation and subsequent immunostaining (Huh et al., 2012; Huh et al., 2013; van der Meer et al., 2013; Maschmeyer et al., 2015; Johnson et al., 2016). In several studies, the secreted cellular products have been analyzed, but they generally lacked the required sensitivity due to the low cell count (~10,000 cells) and the dilution of such products upon continuous perfusion (Huh et al., 2013). Therefore, existing OOCs can be significantly improved by application of a highly sensitive, direct detection tool to assess cytotoxicity, e.g., immediate and *in situ* quantification of the changes in target cell/protein concentrations. It is also preferable to fabricate such a tool as simply and inexpensively as possible to ensure ready availability to the widest selection of researchers.

In order to demonstrate our *in situ*, dual-mode monitoring tool, we propose to prototype a simple OOC with the use of 3D printing and a common house-use cutter machine. We will use a 3D printed template for conventional soft lithography towards fabrication of PDMS-based OOC (Comina et al., 2014), as a simpler and faster alternative. Since 3D printing does not provide sufficient resolution required for promoting cellular adhesion on its surface, we will use a common house-use cutter machine to create the patterns on the inner channel surfaces of an OOC. Although a cutter machine has been previously utilized to “cut” specific channel layouts to fabricate paper-based LOC devices (Fang et al., 2015; Fang et al., 2014), it has not yet been

utilized to modify the surface topography of LOC or OOC devices. This technique can provide a simpler and affordable alternative for adding micro-scale structures to the 3D printed LOC or OOC devices. Such textural detail addition may improve cellular adhesion to the substrates of OOCs.

Most importantly, a non-invasive, *in situ* monitoring tool needs to be incorporated into OOC, which should be easy to use, affordable, and potentially handheld, yet provide accurate and specific assay results. With this goal in mind, we propose the use of a fluorescent nanoparticle immunocapture as well as immunoagglutination assay coupled to a smartphone-based fluorescence microscope. This method will ultimately reduce assay time, offer sufficient assay specificity, ease of fabrication and use, while drastically reducing costly analytical procedures for *in situ* monitoring of cytotoxicity on OOC. Renal proximal tubule derived cells (PTCs) express various PTC specific brush border enzymes, such as γ -glutamyl transpeptidase (GGT), a protein that catalyzes the first step in the metabolism of glutathione (GSH) and GSH conjugates (Tiong et al., 2014). In response to PTC toxicants, the brush border membrane frequently sheds, releasing GGT into the tubular lumen, providing a desired target for detection of cytotoxicity. The use of nanoparticle immunocapture/immunoagglutination for *in situ* monitoring can be incorporated into any existing OOC system by altering the antibody to any given target. Thus, researchers will benefit greatly by improving their OOC analysis techniques. Our report represents the first demonstration of incorporating particle immunocapture/immunoagglutination assays to OOC systems.

Our group has quantified the concentration of bacteria by evaluating the angle-specific Mie scatter signal from immunoagglutinated polystyrene particles (You et al., 2011; Fronczek et al., 2013; Park et al., 2013; Cho et al., 2015). Using smartphone-based optical detection,

Escherichia coli and *Neisseria gonorrhoeae* were sensitively and specifically detected from undiluted human urine, a complicated bodily fluid (Cho et al., 2015). Particle concentration was optimized to detect a varying range of pathogen concentration. Our group has also previously constructed a 3D printed smartphone-based fluorescence microscope for the end-point quantification of PCR products (Angus et al., 2015). As emphasized, existing OOCs and LOCs would benefit greatly from a versatile analysis method that is noninvasive to the device and its content. With the intent of performing analysis on-chip, many approaches have been developed, including electrochemical electrodes, optical sensors, label-free detection of molecules, field-effect transistor sensors, and micro-cantilevers (Sung et al., 2013). One such proof-of-concept study was a portable fluorescence optical detection system to analyze the dynamics of cell viability (Choi et al., 2010). However, the detailed optical system is quite technical and a static 3D culture was fluorescently dyed, which would limit the scope of its application. Cells ideally need to be exposed under flow, especially when studying the cytotoxic response of cells that line directional flow with fluid-filled compartments, which is a similar microenvironment as human tissue.

In the current work, we predicted that the anti-GGT conjugated nanoparticles would be immunoagglutinated upon binding with the GGT either specifically released from the damaged PTCs within the OOC, or captured on the membrane fragments also released from the brush border membrane of PTCs (Fig. 1). Both behaviors can be monitored *in situ* through the use of fluorescent nanoparticles and subsequent detection via a smartphone-based fluorescence microscope. The method provides a novel groundbreaking tool, enabling *in situ*, dual-mode monitoring of the internal and external compartments of the OOC, and which may be integrated into any existing OOCs to facilitate analyses. This dual-mode of detection, i.e., nanoparticle

immunoagglutination and particle capture, has not been previously demonstrated, which is particularly suited for OOC experiments in monitoring both membrane expression and subsequent release of protein products, without the need for collecting, fixing, and/or staining the cells.

2. MATERIALS AND METHOD

2.1 OOC Fabrication

PDMS base and curing agent (Fisher Scientific; Pittsburgh, PA, USA) were combined at 10:1 ratio, and poured over the 3D-printed templates (3.5 mm x 25 mm x 1 mm) adhered to glass Petri dishes (100 mm x 1 mm). The resulting PDMS replicas were separated from the mold, the inlet and outlet holes were made using a 1 mm biopsy punch (Miltex, Inc.; York, PA, USA), and they were bonded to the bottom substrate (microscope glass slide; SPI Supplies; West Chester, PA, USA). The bottom substrate was etched with 400 μm deep line etchings with a Cricut Explore One™ (Provo Craft & Novelty, Inc.; Spanish Fork, Utah, USA). Three layers were patterned onto glass in the following order: straight lines with 20 mm length x 5 mm height with 1 mm spacing in between, zigzag patterns of 20 mm x 10 mm with 15° lines, and zigzag patterns rotated 180° and shifted by 1 mm.

2.2 Antibody Conjugation to Particles

Rabbit polyclonal antibody to γ -glutamyl transpeptidase (anti-GGT; catalog number ab175384, Abcam, Inc.; Cambridge, MA, USA) was used for GGT detection. Bovine serum albumin (BSA; Sigma-Aldrich; St. Louis, MO, USA) was used to generate negative control signals with GGT-positive cell cultures. Anti-GGT and BSA were covalently conjugated to

highly carboxylated, yellow-green, 500-nm diameter, polystyrene fluorescent particles (Masphe, Inc.; Pasadena, CA, USA). The fluorescence characteristics of these particles were reported by the vendor (Magsphere, Inc.) as 480 nm excitation and 510 nm emission. Prior to antibody and BSA conjugation to fluorescent polystyrene particles, particles were centrifuged and pre-washed with deionized water to remove surfactants from the stock solution as received from the vendor. Anti-GGT antibodies and BSA were conjugated to polystyrene particles following the protocol described in Cho et al. (2015).

2.3 Cell Culture and Seeding on OOC

Renal adenocarcinoma cell cultures (786-O; catalog number CRL-1932, ATCC; Manassas, VA, USA) were grown in Roswell Park Memorial Institute medium (RPMI) with L-glutamine (catalog number MT10040CV, Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS; catalog number 10438018, Fisher Scientific). Cells were cultured at 37°C (HERAcell 150i; Cambridge Scientific; Watertown, MA, USA) in 5% CO₂ until 90% confluent.

After reaching ~90% confluency, 786-O cells were passaged following standard procedures (Abcam, 2015). Cells were resuspended at $4.5\text{--}5 \times 10^5$ cells/mL from which 100 μ L aliquots were seeded into each OOC, and incubated in static culture for 24 h. The OOC was subsequently placed in a biosafety cabinet. Polyethylene tubing (Braintree Scientific, Inc.; Braintree, MA, USA) with inner diameter (ID) = 0.381 mm and outer diameter (OD) = 1.092 mm was used to connect the inlet(s)/outlet to 27 gauge needle tips (ID = 0.229 mm and OD = 0.406 mm; Jensen Global; Santa Barbara, CA, USA). Syringes (1 mL; BD, Franklin Lakes, NJ, USA) were connected and positioned on a microfluidic dual syringe pump (New Era Pump Systems, Inc.; Farmingdale, NY, USA).

2.4 Static Assays

Static immunoagglutination assays were performed *ex situ*, i.e., not on OOC, using 786-O lysates, prepared by seeding 60% confluent cells onto 6 cm dishes (Fisher Scientific) for 24 h, 72 h, and 144 h (i.e., until 90-100% confluent). Dishes were washed once with cold DPBS, followed by adding 300 μ L of lysis buffer. Anti-GGT particles or BSA particles were incubated with lysate solutions for 2 h. Static immunoagglutination assay was then performed with a scratched 786-O culture. 786-O cells were cultured and scratched to simulate brush border damage and GGT release by a nephrotoxicant. Microscopic videos of the fluorescent anti-GGT particles and BSA particles were acquired. These assay results were compared with the static GGT activity assay, following the method described by Silber et al. (1986), which measures the rate at which the substrate analog γ -glutamyl-*p*-nitroanilide (GPNA) is cleaved to form *p*-nitroaniline detected spectrophotometrically at 405 nm in a microplate spectrophotometer (Spectramax M2; Molecular Devices; Sunnyvale, CA, USA). In addition, 786-O cell viability assays were conducted on a microplate well, by measuring the optical transmission at 600 nm, with known nephrotoxicant (cisplatin) and non-nephrotoxicant (glycine). Details of all static assays can be found in Supplementary Material 1 – Static Assays.

2.5 Immunoagglutination Assay of the Outflow Solutions from the OOC

Fluorescence light scatter intensities were evaluated on a glass slide (MP Biomedicals; Santa Ana, CA, USA) for the samples (50 μ L) collected from the OOC outlet. Spectral measurements were made using a reflection probe and a pair of optical fibers (R400-7-UV-VIS; Ocean Optics; Dunedin, FL, USA). The 480 nm blue LED irradiated the top of a droplet perpendicularly through the core fiber of a reflection probe, while the backscattered fluorescence

signals were collected through the shell-side bundle of fibers of a reflection probe, which was connected to a miniature spectrometer (USB4000, Ocean Optics) via accompanying software (OceanView; Ocean Optics). Fluorescence of particles was measured at their emission maximum, 515 nm (green). The fluorescence intensities were normalized to those of the BSA particles in the same outflow solutions, or those of the anti-GGT particles in the absence of cells (only DPBS). A normalized intensity value of 1 represented the same fluorescence intensity as that of a normalization reference; an intensity <1 represents the loss of particles, and an intensity >1 represents an increase in fluorescence intensity and evidence of immunoagglutination.

2.6 OOC Immunoagglutination Assay with and without Toxicant

After 24 h incubation, 786-O cells were washed with DPBS, fixed with 4% paraformaldehyde solution (Affymetrix; Cleveland, OH, USA) for 10 min, and then washed with DPBS. A straight-channel OOC (for the assays without toxicant) or a Y-channel OOC (for the assays with toxicant) was used to perform the immunoagglutination assays (using optical fibers and a miniature spectrometer). Cisplatin (known nephrotoxicant) and glycine (known non-nephrotoxicant) were used, at 0, 1, 3 or 5 mM concentrations. Particles in DPBS (1:8 ratio) were passed through a syringe pump at a flow rate of 500 $\mu\text{L/h}$ (without toxicant) or 190 $\mu\text{L/h}$ (with toxicant – lower flow rate was necessary due to the tear in cell monolayer caused by toxicant) for 30 min. For the Y-channel OOC, one inlet was used for toxicant addition while the other for anti-GGT particles. For post-immunoagglutination assays on the OOC, cells were washed once with DPBS for 30 min, and the outflow containing diluted particles were collected into 2 mL tubes (USA Scientific; Ocala, FL, USA). The 786-O cells within the OOC were then fixed with 4% paraformaldehyde solution for 15 min, washed twice with DPBS (500 or 190 $\mu\text{L/h}$), then stored

in DPBS with 0.05% Tween 20 (DPBST; Fisher Scientific). The outflow was collected until the initial DPBS was fully washed away. Assays were repeated with varying concentrations of compounds (glycine and cisplatin), each time using new OOC, under dark conditions to prevent photobleaching of fluorescent particles.

2.7 Fabrication of Smartphone-based Fluorescence Microscope

The smartphone-based fluorescence microscope attachment was designed on SolidWorks software (Dassault Systèmes, SolidWorks Corporation; Waltham, MA, USA) and fabricated using a Dimension uPrint Rapid Prototyping Device (Stratasys, Inc.; Eden Prairie, MN, USA) using acrylonitrile butadiene styrene (ABS) material. Attachment consisted of three white LEDs (Edmund Optics; Barrington, NJ, USA), an objective lens (catalog number CAS100, Thor Labs; Newton, NJ, USA), 480 $\pm$ 10 nm bandpass filter for the light source (catalog number 43-115, Edmund Optics) and 500 nm longpass filter (catalog number GG-495, Edmund Optics) for the smartphone camera. Light sources were powered by two 3 V button batteries (CR2032, Energizer; St. Louis, MO, USA). The smartphone's digital camera (iPhone 5S, Apple, Inc.; Cupertino, CA, USA) functioned as a secondary objective lens as well as an image-capturing device (Fig. 2).

2.8 Smartphone-based *In Situ* Monitoring of On-Chip Expression

All images were collected from the rear glass view of the OOC, which enabled clear image acquisition. Under dark conditions, the OOCs were positioned 10 mm from the primary objective lens of the smartphone-based fluorescence microscope. Images were collected after locking exposure and focus on a blank OOC. With appropriate adjustments to reduce the random

light scatter from etchings of blank OOC, images were collected from various OOCs in presence and absence of cisplatin and glycine. Images were analyzed using ImageJ (National Institute of Health; Bethesda, MD, USA) on a separate desktop computer, and split to red, green, and blue channel images. The green images were assessed to specifically measure the intensity of the green fluorescent signal. On ImageJ, circular areas from the center of the images were analyzed. The fluorescence of immunoagglutinated particles on cell surfaces was measured from these areas of interest. The measured green intensities from OOCs were normalized to the measured green intensity of the blank OOC to eliminate the random light scattering from the etchings within the OOC channel. A series of normalized intensity values were plotted against the concentrations of cisplatin and glycine.

2.9 Statistical Analysis

All reported p-values were obtained from two sample independent t tests of sample versus blank (i.e. BSA-particles or anti-GGT particles with 0 mM toxicant) performed by Stata/IC 12.0 (StataCorp LP, 2011) using $\alpha = 0.05$. Statistical analysis of normalized intensities from outflow solutions were performed comparing cisplatin and glycine per respective concentration.

3. RESULTS

3.1 Static Immunoagglutination Assays of 786-O Lysates

Proximal tubule cells (PTCs) express GGTs on the apical brush border membrane, which typically shed in response to a nephrotoxic insult (Bhise et al., 2014). This shedding causes the release of, and increase in the amount of GGT in the media available for particle

immunoagglutination. Initially, the GGT enzymatic activities from the static 786-O cell monolayer cultures, 24 h, 72 h, and 144 h after seeding, were evaluated by a standard colorimetric enzymatic assay (Silber et al., 1986). A value of 28.4, 35.7, and 44.3 U/L were obtained.

Subsequently, a standard curve was constructed for the immunoagglutination assay with 786-O lysates, with the normalized fluorescent scatter intensities plotted against the GGT enzymatic activities of 28.4, 35.7, and 44.3 U/L. All fluorescent scatter intensities were normalized to those of the anti-GGT particles in the 786-O lysates that were not incubated at all (thus no immunoagglutination). All data points were statistically different from the control (i.e., those that were not incubated), with a linear increase at $y = 0.0054x + 0.99$ ($R^2 = 0.97$) (Fig. 3a).

Next, immunoagglutination assays were repeated for the same 786-O lysates at a fixed GGT activity, 31.8 U/L, but this time using BSA conjugated particles in comparison with anti-GGT particles. The normalized intensity with BSA particles is very close to 1, which represents the same fluorescence intensity of the BSA particles with or without incubation (Fig. 3b). There is a statistically significant difference of the normalized intensity with BSA particles than with anti-GGT particles (Fig. 3b). Thus, BSA particles can be used as a good negative control in the presence of tissue culture media and other cellular contaminants.

Microscopic images of anti-GGT particles revealed large aggregates with increased fluorescence (Fig. 3c), whereas the equivalent BSA particles revealed primarily singlets, i.e. non-agglutinated (Fig. 3d). In addition, the majority of immunoagglutinated anti-GGT particles were identified on the cell surface, whereas the BSA particles did not bind to the apical cell surface.

3.2 OOC Assays in the Absence of Toxicant

After 24 h incubation of 786-O cells in the OOC under static flow conditions, immunoagglutination assays were conducted on the outflow from the OOC at 500 $\mu\text{L}/\text{h}$ for 30 min. Two single, straight channel OOCs were used in parallel to evaluate the immunoagglutination assays using anti-GGT particles in one channel, and BSA particles in the other, under identical conditions. All fluorescence scatter intensities were normalized to the experiments replicated with DPBS (normalization reference), with respective particle type (anti-GGT or BSA particles). Anti-GGT particles showed a 15% decrease in normalized intensity from the control (Fig. 4a), which correlates to a loss of particles in the outflow. Following the same protocol, BSA particles exhibited a slightly increased normalized intensity from the control (Fig. 4a). This slightly higher intensity may arise from the non-specific aggregation of BSA particles, under the long duration of assay time (1 h). Since there was a loss of signal in the outflow with anti-GGT particles, we suspect that the particles are bound to the cell surface and subsequently trapped within the OOC. This interpretation was confirmed with the fluorescence microscopic images of the chip surface showing greater and sharper fluorescence and the presence of particles on the chip surface (Fig. 4c). In contrast, the BSA particles appeared more diffused, and were not firmly attached to the chip surface (Fig. 4b), but remained discharged into the outflow.

3.3 Chemical-Induced Cytotoxicity Assays in OOC

Immunoagglutination assays for the outflow solutions from OOC were performed with the 24 h cultured 786-O cells incubated with either glycine or cisplatin. Fluorescence scatter intensities were normalized as described above with particles diluted in DPBS. The results followed the pattern observed in Supplementary Material 1 – Static Assays. At varying glycine

concentration, the normalized intensities remained similar, at ~50% of the reference (DPBS) (Fig. 5), indicating that ~50% of anti-GGT particles remained bound to the 786-O cell surface within the OOC and that glycine had no effect on altering such GGT expression. In contrast, increasing concentrations of cisplatin, raised normalized intensities from 0.50 (no cisplatin), 0.63 (1 mM cisplatin), 1.43 (3 mM cisplatin), and 1.90 (5 mM cisplatin) (Fig. 5). In other words, it varied from the 50% loss of anti-GGT particles (no cisplatin) to the 90% increase by immunoagglutination (5 mM cisplatin). These results indicate that with increasing cisplatin concentration the anti-GGT particles no longer remain bound to the cell surface within the OOC and are immunoagglutinated with the GGTs shed into the outflow solutions. These results are consistent with the fact that 786-O cell viability decreases at increasing cisplatin concentrations (Supplementary Material 1 – Static Assays).

3.4 *In Situ* Monitoring with Smartphone-based Fluorescence Microscopy

A smartphone-based fluorescence microscope was used to capture images of the OOC channels *in situ*, to monitor treatment induced cytotoxicity. Normalized intensities were collected in the same manner as described above (normalized to the respective particles diluted in DPBS). Subsequent analysis revealed the extent of immunoagglutination between the anti-GGT particles and the GGTs on the cell surface within the OOC. Smartphone-based *in situ* monitoring on OOC revealed no significant changes in normalized intensities at different glycine concentrations, and were similar to that in absence of glycine (0 mM) (Fig. 6a). This is consistent with the lack of toxicity of glycine. In contrast, 1 mM cisplatin enhanced the normalized intensity relative to the 0 mM control, a consequence of GGT release from the brush border membrane in response to a cytotoxicant. Higher concentrations of cisplatin (3 and 5 mM) resulted in a linearly decreasing

trend (Fig. 6), which is similar to that observed in the static cell viability (Supplementary Material 1 – Static Assays) and OOC assays (Fig. 4). Overtly cytotoxic concentrations of cisplatin causes cell death and cell detachment from the OOC surface in a manner equivalent to physiological anoikis, resulting in reduced amounts of anti-GGT particles bound to the chip surface and a decrease in normalized intensities. These findings are also consistent with the increased normalized intensities observed from the OOC outflow under high cisplatin concentrations (Fig. 5).

In situ images acquired by the smartphone-based fluorescence microscope from the surface of the OOC are shown in Fig. 6b, and revealed that at 0 mM glycine or cisplatin no significant fluorescence was observed. However, significant fluorescence was observed at 5 mM glycine, perhaps explaining the larger error bar for 5 mM glycine in Fig. 6a. Substantial fluorescence was observed with 1 mM cisplatin, consistent with the data in Fig. 6a. Similarly, 5 mM cisplatin diminished fluorescence consistent with other data (Figs. 4 and 6a; Supplementary Material 1).

4. DISCUSSION

786-O cell cultures exhibit markers of differentiation representative of renal proximal tubule cells (PTCs) (Chiatar et al., 2013). We confirmed the presence of GGT enzymatic activity in 786-O cell lysates from 24 h, 72 h, 144 h cultures (28.4, 35.7, and 44.3 U/L). A standard curve was constructed, which demonstrated an increase of statistically significant fluorescent scatter intensities with increasing GGT enzymatic activity (Fig. 3a). Existing studies show that the detection limit of 1 U/L GGT can be measured from human urine by following standard automatic biochemistry analyzer (Zhang et al., 2015). However, the values tested in this study

(28.4 – 44.3 U/L) covered the normal range of GGT expression (Orlowski and Szeqczuk, 1962; Westhuyzen et al., 2003, Zhang et al., 2015), which our anti-GGT particles may indeed detect and monitor the changes in GGT enzymatic activity. Meanwhile, higher level of GGT expression is expected under nephrotoxic insult. A previous clinical study reported a significant contrast of urinary GGT concentration among patients without acute renal failure (40.3 U/L) versus patients with the condition (77 U/L; Westhuyzen et al., 2003). Ideally, the standard curve can be used to quantify the GGT expression in the collected outflow solution of a nephrotoxic-induced organ-on-a-chip in order to expedite early detection in lieu of measuring excreted GGT from urinary samples.

The static immunoagglutination assay revealed that anti-GGT particles and subsequent fluorescence scatter measurements could successfully detect the presence of GGT in these lysates. BSA particles served as an appropriate negative control since they do not immunoagglutinate in the presence of GGT. To compare the extent of immunoagglutination between BSA particles and anti-GGT particles, 786-O monolayers cultured for 24 h were scratched to induce shedding of brush border membrane proteins in a fashion similar to that seen in response to nephrotoxicants. Incubation (2 h) of BSA particles with the pre-scratched 786-O monolayers resulted in the presence of primarily randomly drifting particles (Supplementary Video S1). In contrast, incubation (2 h) of the anti-GGT particles with the pre-scratched 786-O cell monolayers resulted primarily in fixed particles on the cell surface, as would be anticipated by the capturing of anti-GGT particles on the brush border membrane (Supplementary Video S2). Anti-GGT particles also bound to the scratched surface, probably due the presence of cell fragments that remain adhered to the glass surface. Normalization was performed with the particles diluted in lysate solution, and consisted primarily of singlets. This provided a precise

normalization rather than utilizing a DPBS solution since the lysates themselves induce non-specific aggregation and thus contribute to the increase in scattering signal. (Later experiments used DPBS as a negative control since lysates were not used.) Under the flow conditions using an OOC (with no toxicant), the outflow was assayed for GGT using anti-GGT and BSA particles (as control). With BSA particles, a slight increase in normalized intensity was observed, resulting from minor, non-specific, aggregation due to the long assay time (1 h). With anti-GGT particles, a significant decrease in normalized intensity was observed since 786-O cells express GGT on their brush border membrane, which captures the anti-GGT particles within the OOC, reducing the amount of GGTs in the outflow.

When investigating chemical- and drug-induced nephrotoxicity under the flow conditions of the OOC, the analysis of outflow and the *in situ* monitoring by a smartphone-based fluorescence microscope offered a coupled-tool for validating the assay in two simultaneous ways. As a nephrotoxicant (cisplatin) circulated through the OOC, GGT release into the media from 786-O cells increases, with corresponding increases in immunoagglutination of the anti-GGT particles in the outflow. Thus, as nephrotoxicant concentrations increase, GGT availability and subsequently normalized intensity increase in the outflow (see Fig. 5, inset). In contrast, *in situ* monitoring within the OOC revealed a complementary decrease in normalized intensity, due to the loss of cells at overt cytotoxicity (Fig. 6). The dynamic range of the system thus benefits from this inversely coupled assay approach, as intensity in the outflow increases, whilst the intensity within the OOC decreases.

In response to drug-induced nephrotoxicity, all static and OOC assays with glycine exhibited no significant changes, consistent with glycine being non-toxicant. In contrast, studies with cisplatin exhibited significant increases in signal in the outflow and a general decrease in

signal within the OOC, as expected from a known nephrotoxicant. The use of glycine and cisplatin therefore confirms the specificity of the assays. It may be possible that glycine and cisplatin had some effects on particle stability and thus generating non-specific signal increase. Therefore, both anti-GGT and BSA particles were incubated with 5 mM glycine or 5 mM cisplatin, and no significant signal increase was observed (Supplementary Material 2 – Effect of Toxicant on Particle Stability).

5. CONCLUSION

No previous reports addressing the incorporation of particle immunoagglutination assays into the OOC have yet been published. Standard methods of analyzing kidney cell culture upon exposure to toxicants include cell viability assay, enzymatic assay of the released GGTs, and fluorescence immunostaining of the GGTs expressed on cell membranes (Wilmer et al., 2016). The results of our particle immunoagglutination assay matched very well to those of static cell viability assay (Supplementary Material 1 vs. Figure 6A – trends are almost identical) as well as the GGT enzymatic assay (Figure 3A showed excellent correlation with $R^2 = 0.9664$ between immunoagglutination and GGT enzymatic assays). As for immunofluorescence staining, our fluorescent anti-GGT nanoparticles perform similarly to the fluorescent dyes of immunofluorescence staining, while they provided additional advantage of dual-mode monitoring of GGT. Since particle immunoagglutination assays and subsequent light scatter detection can serve as an essentially one-step, rinse-free assay with very low limits of detection (typically down to 10 pg proteins per mL sample) (You et al., 2011; Fronczek et al., 2013; Park et al., 2013; Cho et al., 2015), this method can serve as an easy-to-use and versatile monitoring tool for OOC. Additionally, the antibody-conjugated particles can be used for monitoring both

the outflow and the on-chip target protein content in response to induced cytotoxicity, offering a coupled-tool for validating the assays in two simultaneous ways. Furthermore, the inverse relationship of the coupled assays significantly increases the overall dynamic range of the system. Such unique and advantageous tool can be used towards more complex OOCs, for example, those requiring co-culture of different cell types. Co-culture has widely been considered a necessary trait in reconstituting the true organ-level functionality. Our dual-mode detection enables efficient analysis *in situ*, while with conventional invasive methods there is a risk of cross-contamination and disruption of *in vivo* environment. We also demonstrated the application of a 3D printed template and a commercial cutter machine to provide a simple and affordable fabrication of OOC. Moreover, the versatility of the immunoagglutination method also facilitates the testing of any protein for which antibodies are available to target. The *in situ* monitoring of the extent of immunoagglutination within the OOC with a smartphone-based microscope, another very important aspect of this work, and to the best of our knowledge is novel. Not only does such a hand-held, compact, and feasible analytic device simplify the analytic monitoring within the OOC (and potentially other LOCs), but it can broaden research capabilities for those who otherwise lack the resources. The fast growing smartphone market in the developing world has enabled their potential application for rapid diagnostics (Yetisen et al., 2013).

Literature survey on recent nephrotoxicity studies with biosensor emphasis revealed that electrochemical sensors have been majorly utilized. In addition, cell viability and/or proliferation has been unanimously evaluated in such work (Cai et al., 2014; Lei et al., 2014; Liu et al., 2013; Shih et al., 2013; Tran et al., 2013; Zan et al., 2013), which assessed only the presence/absence of cells, and did not concern the analysis of cellular response. Such methodologies may satisfy

initial drug screening and cell viability assay (Lei et al., 2014; Shih et al., 2013; Tran et al., 2013), but will not be able to provide detailed insight regarding cellular response. Thus, the incorporation of our dual-mode, protein marker-specific nanoparticle immunoagglutination assay within organ-on-a-chip may greatly potentiate the emergence of biosensors towards detailed drug screening and the study of cellular responses. Future direction of our research may entail investigations on detection from multiple target presence (using monoclonal antibodies), use of other enzyme/protein markers, not limited to nephrotoxicity, and to collaborative opportunities to incorporate our novel analytic methodologies to existing OOC/LOC systems.

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REFERENCES

- Abcam, 2015. Cell Culture Guidelines, Abcam, Inc., Cambridge.
<http://www.abcam.com/ps/pdf/protocols/cell_culture.pdf> (accessed 26.10.2015).
- Angus, S.V., Cho, S., Harshman, D.K., Song, J.Y., Yoon, J.Y., 2015. Biosens. Bioelectron. 74, 360-368.
- Bhise, N.S., Ribas, J., Manoharan, V., Zhang, Y.S., Polini, A., Massa, S., Dokmeci, M.R., Khademhosseini, A., 2014. J. Control. Release 190, 82-93.

Cai, H.-H., Pi, J., Lin, X., Li, B., Li, A., Yang, P.-H., Cai, J., 2015. *Biosens. Bioelectron.* 74, 165-169.

Chiatar, S.S., Eze, O.P., Schoenfeld, A.R., 2013. *Cell Dev. Biol.* 2, 1-12.

Cho, S., Park, T.S., Nahapetian, T.G., Yoon, J.Y., 2015. *Biosens. Bioelectron.* 74, 601-611.

Choi, J.R., Sung, J.H., Shuler, M.L., Kim, D., 2010. *Optics Lett.* 35, 1374-1376.

Comina, G., Suska, A., Filippini, D., 2014. *Lab Chip* 14, 424-430.

Esch, M.B., Sung, J.H., Yang, J., Yu, C., Yu, J., March, J.C., Shuler, M.L., 2012. *Biomed. Microdev.* 14, 895-906.

Fang, X., Guan, M., Kong, J., 2015. *RSC Adv.* 6, 64614-64616.

Fang, X., Wei, S., Kong, J., 2014. *Lab Chip* 14, 911-915.

Fronczek, C.F., You, D.J., Yoon, J.Y., 2013. *Biosens. Bioelectron.* 40, 342-349.

Ghaemmaghami, A.M., Hancock, M.J., Harrington, H., Kaji, H., Khademhosseini, A., 2012. *Drug Discov. Today* 17: 173-181.

Huh, D., Kim, H.J., Fraser, J.P., Shea, D.E., Khan, M., Bahinski, A., Hamilton, G.A., Ingber, D.E., 2013. *Nat. Protoc.* 8, 2135-2157.

Huh, D., Leslie, D.C., Matthews, B.D., Fraser, J.P., Jurek, S., Hamilton, G.A., Thorneeloe, K.S., McAlexander, M.A., Ingber, D.E., 2012. *Sci. Transl. Med.* 4, 159ra147.

Jang, K.J., Mehr, A.P., Hamilton, G.A., McPartlin, L.A., Chung, S., Suh, K.Y., Ingber, D.E., 2013. *Integr. Biol.* 5, 1119-1129.

Johnson, B.N., Lancaster, K.Z., Hogue, I.B., Meng, F., Kong, Y.L., Enquist, L.W., McAlpine, M.C., 2016. *Lab Chip*, 16, 1393-1400.

Lei, K.F., Wu, M.-H., Hsu, C.-W., Chen, Y.-D., 2014. *Biosens. Bioelectron.* 51, 16-21.

- Liu, J., Qin, Y., Li, D., Wang, T., Liu, Y., Wang, J., Wang, E., 2013. *Biosens. Bioelectron.* 41, 436-441.
- Maschmeyer, I., Lorenz, A.K., Schimek, K., Hasenberg, T., Ramme, A.P., Hübner, J., Lidner, M., Drewell, C., Bauer, S., Thomas, A., Sambo, N.S., Sonntag, F., Lauster, R., Marx, U., 2015. *Lab Chip* 15, 2688-2699.
- Orlowski, M. and Szeqczuk, A., 1962. *Clin. Chim. Acta* 7, 755-760.
- Park, T.S., Li, W., McCracken, K.E., Yoon, J.Y., 2013. *Lab Chip* 13, 4832-4840.
- Shih, S.C.C., Barbulovic-Nad, I., Yang, X., Fobel, R., Wheeler, A.R., 2013. *Biosens. Bioelectron.* 42, 314-320.
- Silber, P.M., Gandolfi, A.J., Brendel, K., 1986. *Anal. Biochem.* 158, 68-71.
- StataCorp LP, 2011. *Stata/IC 12.0* <<http://www.stata.com/>> (accessed 06.11.16).
- Sung, J.H., Esch, M.B., Prot, J.M., Long, C.J., Smith, A., Hickman, J.J., Shuler, M.L., 2013. *Lab Chip* 13, 1201-1212.
- Tiong, H.Y., Huang, P., Xiong, S., Li, Y., Vathsala, A., Zink, D., 2014. *Mol. Pharmaceut.* 11, 1933-1948.
- Tran, T.B., Cho, S., Min, J., 2013. *Biosens. Bioelectron.* 50, 453-459.
- van der Meer, A.D., Orlova, V.V., ten Dijke, P., van den Berg, A., Mummery, C.L., 2013. *Lab Chip* 13, 3562-3568.
- Westhuyzen, J., Endre, Z.H., Reece, G., Reith, D.M., Saltssi, D., Morgan, T.J., 2003. *Nephrol. Dial. Transplant* 18, 543-551.
- Wilmer, M.J., Ng, C.P., Lanz, H.L., Vulto, P., Suter-Dick, L., Masereeuw, R., 2016. *Trends Biotechnol.* 34, 156-170.
- Yetisen, A.K., Akram, M.S., Lowe, C.R., 2013. *Lab Chip* 12, 2210-51.

You, D.J., Geshell, K.J., Yoon, J.Y., 2011. Biosens. Bioelectron. 28, 399-406.

Zan, X., Fang, Z., Wu, J., Xiao, F., Huo, F., Duan, H., 2013. Biosens. Bioelectron. 49, 71-78.

Zhang, Y.-R., Wang, P., Liang, X.-X., Tan, C.S., Tan, J.-B., Wang, J., Huang, Q., Huang, R., Li, Z.-X., Chen, W.-C., Wu, S.-X., Ong, C.N., Yang, X.-F., Wu, Y.-N. 2015. Int. J. Environ. Res. Public Health 12, 11988-12001.

FIGURE CAPTIONS

Figure 1. Schematic illustration of dual-mode detection. Anti-GGT particles bind to the GGTs expressed on the membrane of 786-O proximal tubule cells (PTCs), which can be identified *in situ* (i.e. on chip). With nephrotoxic treatment, such GGTs are released from the membrane and detected in the outflow solution.

Figure 2. *In situ* monitoring OOC device. A smartphone-based fluorescence microscope measured fluorescence scatter intensities from the Y-channel OOC. Red-dyed water was flown through the OOC for the purpose of visualization.

Figure 3. Standard curve of static immunoagglutination GGT assay from the 786-O lysates. A standard curve was constructed from a series of particle immunoagglutination GGT assays from the 786-O lysates under static conditions for 2 h, where x-axis is the enzymatic activities of GGT (a). Scatter intensities were normalized to those that have not been incubated (i.e., comprised of mostly singlets). The results with BSA conjugated particles are also shown in (b). Fluorescence imaging on a light microscope showed aggregates and increased intensity from the 786-O lysates with anti-GGT particles (c), in contrast to majority of singlets from the 786-O

lysates with BSA particles (d). * denotes $p < 0.05$ of two sample independent t-test; ** denotes $p < 0.0001$ of two sample independent t-test.

Figure 4. Particle immunoagglutination assay of the outflow solutions from OOC without toxicant. Compared to the normalized fluorescence scatter intensity with BSA particles, those with anti-GGT particles was lower by about 20% (a). Fluorescence scatter intensities of anti-GGT particles and BSA particles were normalized to the fluorescence scatter intensity of respective particles diluted in DPBS without incubation. Fluorescence imaging of the OOC channel using a light microscope showed greater presence of particles and fluorescence with anti-GGT particles (c), than BSA particles (b). * denotes $p < 0.05$ of two sample independent t-test.

Figure 5. Particle immunoagglutination assay for the outflow solutions from OOC with toxicants. With increasing glycine concentration, the normalized intensities stayed the same around 0.5. With increasing cisplatin concentration, the normalized intensities increased. Inset images show the illustrations of anti-GGT particles with toxicant treatment. With no toxicant or glycine, GGT antigens are intact on the 786-O membranes, which renders anti-GGT particles to remain largely as singlets in the outflow solution. With increasing cisplatin concentrations, GGT antigens are released into the media and subsequently to the outflow solution as cell viability decreases. Anti-GGT particles more easily bind to free GGT antigens under flow condition, rather than binding to the disturbed membrane. With 5 mM cisplatin, hardly any cells are present within OOC. With increasing GGT antigens, extent of anti-GGT particle immunoagglutination

increases, which contributes to the highest normalized intensities. ** denotes $p < 0.0001$ of two sample independent t-test.

Figure 6. *In situ* assay results with smartphone-based fluorescence microscope. Images were taken after 1 h immunoagglutination assay in OOC with induced nephrotoxicity (glycine vs. cisplatin). Normalized fluorescence scatter intensities, obtained from image analysis, are plotted against varying concentrations of glycine (non-nephrotoxicant) and cisplatin (PTC-specific nephrotoxicant) (a). *In situ* images taken by the smartphone-based fluorescence microscope with 0 mM (absence of toxicant), 1 mM glycine, 5 mM glycine, 1 mM cisplatin, and 5 mM cisplatin (b). Images are acquired with 5x magnification. * denotes $p < 0.05$ of two sample independent t-test.

Supplementary Material 1. Static Assays.

Supplementary Material 2. Effect of Toxicant on Particle Stability.

Supplementary Video S1. Video of static incubation of the BSA particles and the 786-O cells under scratched conditions. 786-O cells, after 24 h incubation, were scratched with a pipette tip to induce damage of brush border membrane. The well plate was slightly vibrated to demonstrate the floating BSA particles that were neither immunoagglutinated nor bound on the cells. Video is sped up 3x that of original.

Supplementary Video S2. Video of static incubation between anti-GGT particles and cells under scratched conditions. 786-O cells, after 24 h incubation, were scratched with a pipette tip

to induce shedding of brush border membrane. Video demonstrated that the majority of anti-GGT particles were fixed on the surface. Video is sped up 3x that of original.