LIVE QUANTIFICATION OF CELL VIABILITY VIA NEUTRAL RED UPTAKE USING LENS-FREE IMAGING

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ABSTRACT

We present the quantification of cell viability during neutral red (NR) uptake with a compact lens-free system utilizing two light sources. Conventionally, the NR uptake assay determines cell viability based on the accumulation of NR within lysosomes and is quantified spectrophotometrically after a destructive extraction process. Our NR uptake live imaging system offers *in situ* monitoring of cell viability while accentuating 3D morphologies, thus diversifying the assay's functionality. Combining the low-cost NR uptake assay with the compact architecture of lens-free imaging provides high-throughput, point-of-use, live monitoring of cell viability that eliminates extraction steps and preserves the culture.

KEYWORDS: Lens-free imaging, Cell Viability, 3D Culture, Hepatic Cancer Cells

INTRODUCTION

Determining cell viability for *in vitro* tissue culture is crucial for evaluating cell health, toxicity of compounds, and efficacy of therapeutics. NR is a common and inexpensive supravital stain for assessing cytotoxicity. Healthy cells accumulate NR as the nonionic dye diffuses into an acidified lysosome and becomes protonated. In the standard assay, cell viability is quantified spectrophotometrically as an ensemble average of the culture after destructive extraction of the accumulated dye.[1] Lens-free imaging provides real-time culture monitoring at a lower cost, wider field of view, and smaller footprint for high-throughput, point-of-use, quantitative live-cell imaging. In lens-free imaging, a light source directly illuminates the sample that is within immediate proximity of the CMOS sensor array. The result is a shadow image with the size and resolution of the array. We present the use of lens-free imaging to monitor viability of human liver cancer cells during NR uptake, rather than after extraction.

EXPERIMENTAL

Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the NIST, nor does it imply that the materials or equipment is necessarily the best available for the purpose.

Culture vessels were created by plasma bonding poly-dimethylsiloxane (PDMS) wells or micropatterns to #1 glass coverslips, then sterilized with 70% ethanol. Flow in microchannels (400 µm wide, 70 µm high, 30 mm long) was controlled with a gentle gravity flow (Δh_{max} = 0.9 cm). A hybrid cell adhesion matrix (HCAM) was prepared on the surfaces with 50 mg/L fibronectin in Dulbecco's phosphate buffered saline followed by 1 g/L poly(allylamine) in deionized water.

Human hepatoma cells (HepG2) were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (DMEM) at 37 ºC and 5% CO_2, harvested at 80% confluency, seeded in culture vessels at a 1:1 vessel:flask surface density and stained within (5 to 6) d. The lens-free system (SOL Inc., KR) has an 8 megapixel CMOS sensor (field-of-view = 10.14 mm^2, spatial resolution = 1.10 µm, pixel size = 1.12 µm). Light sources were filtered at 540±35 nm. The NR uptake assay was performed similar to the standard protocol.[1] After pre-assay imaging, dye-free DMEM was replaced with 40 mg/L NR in DMEM and every hour thereafter for 3 h. Final images were collected with dye-free DMEM. Images were analyzed with

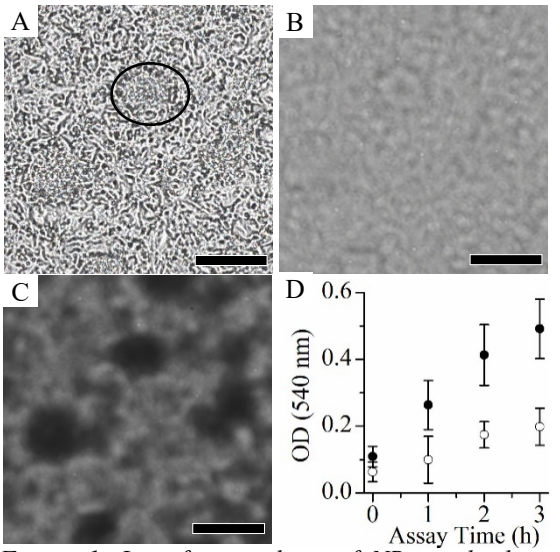

Figure 1. Lens-free analysis of NR uptake by a confluent culture. (A) Pinhole light at 0 h. Collimated light at (B) 0 h and (C) 3 h. (D) NR uptake by clusters (solid) and monolayers (open). Scale bar = 200 µm.

Image J[2]. Briefly, pinhole-illuminated images were used to define regions for analysis by subcategorizing cell into "Dense clusters" (circular bodies not in excess of 75 pixels [91 μm^2] or "Spread monolayers" (oblong bodies up to 500 pixels [665 μm^2]). For confluent cultures, areas of consistent cell morphology were marked with circular regions of 1976 pixels (2390 μm^2). Marked regions were transferred to respective collimated-light images to determine the transmittance and calculate absorbance.

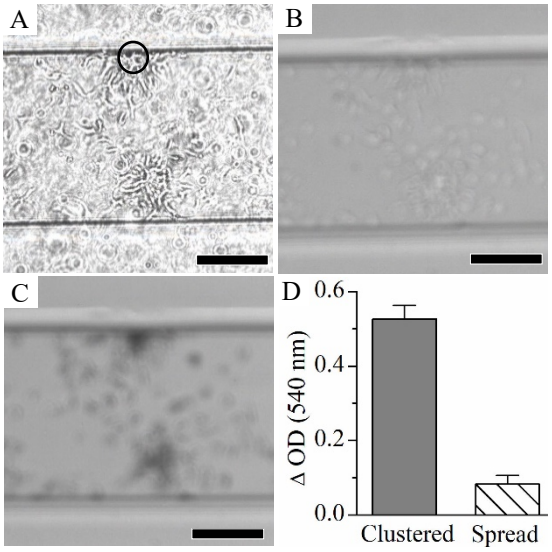

Figure 2. Lens-free analysis of NR uptake by HepG2 cells in a microfluidic channel. (A) Pinhole light at 0 h. Collimated light at (B) 0 h and (C) 3 h. (D) Absorbance of NR in cells. Scale bar 200 μm.

RESULTS AND DISCUSSION

The lens-free imaging resolves cells when using a pinhole light source due to cell perimeter scattering light that appear darker and the cell bodies focusing light, frequently saturating the pixel below. This artifact is rendered negligible with a collimated light source (Fig. 1A and B). Thus, imaging occurs in tandem with 1) the pinhole source locating cells and, 2) the collimated source quantifying optical density. Two distinct morphologies are observed herein: 1) spread monolayers, or 2) dense clusters. Under pinhole illumination, dense clusters (Fig. 1A oval) are darker and appear to organize into 3D morphologies similar to multicellular spheroids previously observed on HCAM[3].

HepG2 cells steadily accumulate NR over the 3 h assay (Fig. 1C and 1D), consistent with the standard NR uptake assay[1]. The dense clusters appear to uptake more NR than spread monolayers. The disparity between morphologies may be due to a difference in pathlength caused by an increase in cell height. Previous work demonstrates that HCAM stimulates HepG2 cells to form 3D cultures[3]. Confocal images of fixed cultures confirm the heights of dense clusters vary from (15 to 35) μm, whereas spread monolayers vary from (4 to 6) μm.

The elimination of the extraction process enables the NR uptake assay to quantify cell viability in microfluidics. Despite the presence of debris within the PDMS, cell perimeters are still identifiable with the pinhole source (Fig. 2A) and NR accumulations is unobstructed with collimated light (Fig. 2B and 2C). Spread cells exhibit a greater cell area while clustering cells (Fig. 2A circle) are more confined and spherical. Clustered cells absorb more light than the spread cells (Fig. 2D), likely due to concentration in lysosomes caused by a reduced cell area.

CONCLUSION

Tandem lens-free imaging with pinhole and collimated light sources enables *in situ* quantification of cell viability and reduces the time to verification for the NR uptake assay. Additionally, NR uptake visually enhances 3D cell morphologies. The reduced footprint of lens-free imaging combined with low-cost supravital stains like NR enables high-throughput assessment of cell viability on a cell-by-cell basis at the point-of-use (e.g., incubator) where traditional microscopes would be disruptive, inhibitory, or costly.

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REFERENCES

[1] Repetto G.; del Peso A.; Zurita J.L., *Nat. Protoc.*, 3, 1125-1131, 2008.
[2] Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W., *Nat. Meth.*, 9, 671-675, 2012.
[3] Bhadriraju, K.; Hong, J.S.; Lund, S.P.; Reyes, D.R., *ACS Biomater Sci Eng.*, 3, 2559-2569, 2017.

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