Lens-Free Imaging as a Sensor for Dynamic Cell Viability Detection Using the Neutral Red Uptake Assay

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ABSTRACT: Neutral red is a low-cost supravital stain for determining cell viability. The standard protocol relies on a destructive extraction process to release the accumulated dye for endpoint spectrophotometric quantification. We report a non-destructive, live-cell quantification of neutral red uptake using a compact lens-free system. Two light sources indentify the cell perimeter and quantify neutral red uptake. The quantification occurs during staining, thus eliminating the destructive extraction process and reducing assay time. Our system enables live quantification for continuous high-throughput screening of cell viability within confined spaces such as incubators.

In-vitro cell viability assays are crucial for quantifiying the toxicity of experimental therapeutics[1,2], toxins[3], and environmental contaminants[4-7] Common *in-vitro* cell viability assays involve supravital stains which oxidize NAD(P)H-dependent enzymes to provide relative metabolic activity (e.g., tetrazole MTT and resazurin reduction assays)[8-10], bind to proteins for determining relative protein content (e.g., sulforhodamine B and Kenacid Blue assays)[11-13], or sequester within organelles for evaluating cellular integrity (e.g., neutral red uptake assay (NRUA))[14, 15].

The NRUA was initially developed to visualize and quantitatively estimate immunotoxicity and viral cytopathogenicity[16], later adopted as a high-throughput cytotoxicity assay[14], then accepted as an alternative to animal testing[17] and assessment for phototoxicity[18]. The NRUA identifies healthy cells as they accumulate neutral red (NR) when the nonionic dye becomes protonated upon entering an acidified lysosome. Non-viable cells release the entrapped NR due to a disruption in lysosomal acidity or loss of cellular integrity.[19] An extensive extraction process resolubilizes the accumulated dye from healthy cells for spectroscopic quantification.[15] The result is an ensemble average of relative cell viability over the entire cell culture. Cytotoxicity of a compound is estimated by testing multiple doses in 96-well plates and comparing their relative viability to that of an untreated negative control. Like other supravital stains, the NRUA requires destructive post-processing for quantification and precludes monitoring dynamic behavior of the cell culture during the assay.

Live quantitative imaging provides a non-destructive assessment of morphology and viability without the need for post processing (Fig. 1). Traditional optical microscopes offers a wide variety of techniques and excellent image quality. Their bulky housings, however, complicate continuous live imaging of cell cultures within confined spaces. Reducing the form factor of microscopes enables their integration into safety cabinets and incubators for high-throughput, real-time monitoring of cell cultures and assays. Lens-free imagers offer compact housings and a wider field-of-view than traditional optical microscopes. In lens-free microscopy (Fig. 2), a light source directly illuminates a sample

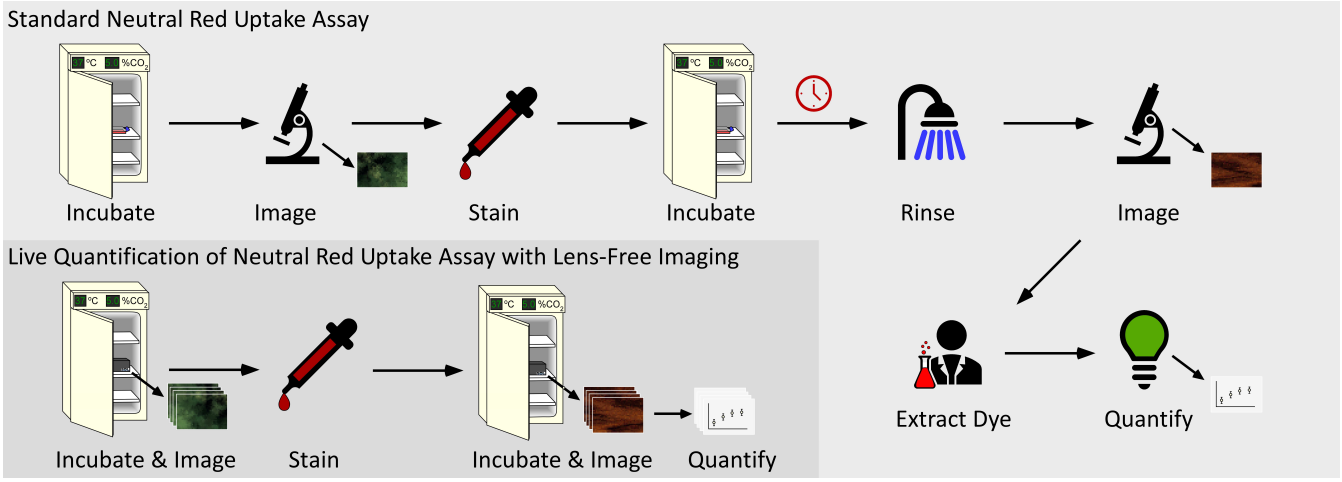

Figure 1. Procedural advantage of live quantitative image analysis versus the standard NRUA.

typically placed within immediate proximity of a sensor array.[20] A shadow image is generated with a size and resolution of the pixel array. By varying source wavelength, source positioning and/or sample height, the shadow can be enhanced with holographic image reconstruction techniques to quantify size, shape, and viability of microparticles and cells on 2-D surfaces or in 3-D suspensions.[21-24]

Herein, we demonstrate a simple lens-free live-imaging method for *in-situ* quantification of the NRUA. In this new approach, the lens-free system images and quantifies cell viability as the cells accumulate NR, rather than after a destructive chemically extraction of the dye. To accomplish this, the lens-free system utilizes two light sources (Fig. 2): 1) a pinhole source for defining regions of analysis via contrast imaging of the cell perimeter and 2) a color-filtered collimated source that minimizes optical artifacts to precisely quantify the transmitted light above each pixel. This tandem lens-free imaging quantifies NR during uptake without extensive data collection and computational resources required for holographic image reconstruction. In addition, *in-situ* imaging accentuates areas of 3-D growth within the culture. As exemplified with human liver cancer cells (HepG2), regions of 3-D growth absorb more light than monolayers, which yields the live NRUA as an inexpensive method for screening conditions that stimulate 3-D growth. The combination of lens-free imaging and the NRUA provides a rapid high-throughput *in-situ* screening method for cell viability and 3-D growth with a form factor that accommodates the spatial limitation of incubators.

Phase contrast or differential interference contrast methods are typically required to adequately resolve cells with transmitted light microscopy. For the lens-free system presented herein, imaging is optimized by minimizing the separation distance between the sample and imaging sensor (Fig. 2). With pinhole illumination, cell perimeters tend to scatter light, thus appearing darker, while the cell bodies behave as a lens to focus light and frequently saturate the pixel below (Fig. 3A and Fig. S1 in Supporting Information). Similarly, any non-uniformity in the materials also cast a shadow image to generate artifacts (Fig. 3 arrows). Switching to a collimated light source, however, significantly reduce the scattering and lensing effects of the cell body while minimizing

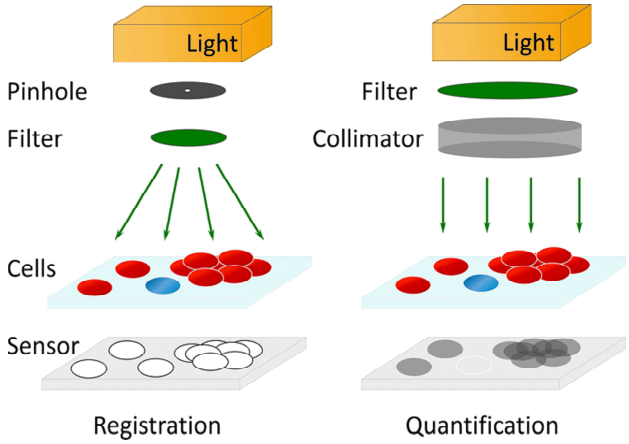

Figure 2. Schematic of lens-free imaging with pinhole and collimated light sources. Pinhole-illumination provides location data used for quantifying absorbance from images with collimated illuminatation.

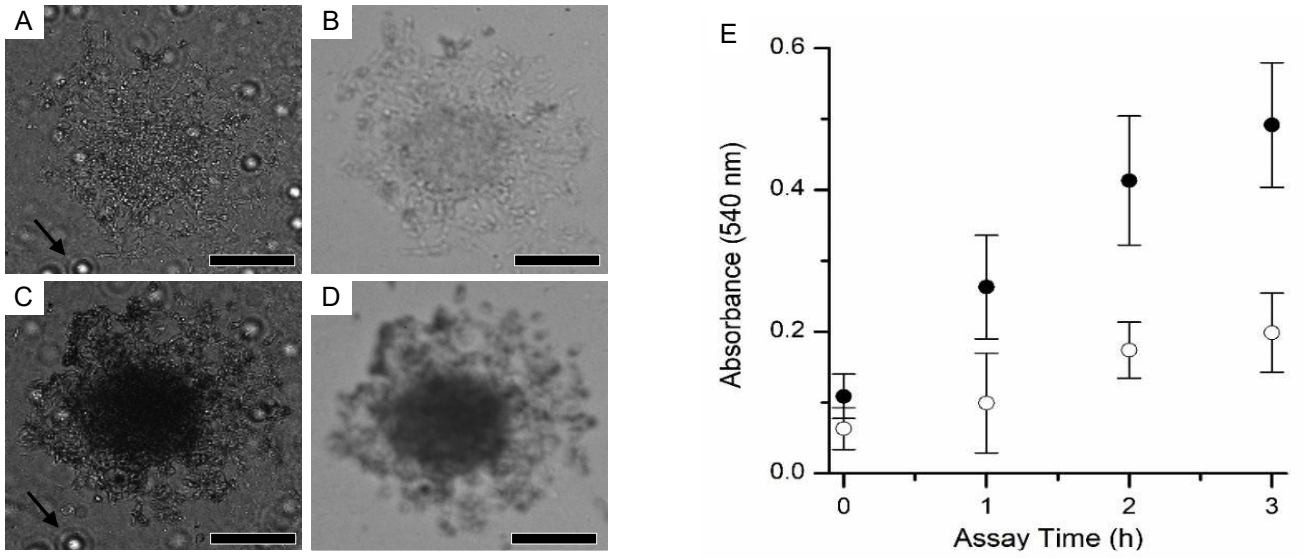

Figure 3. Lens-free monitoring of NR uptake by HepG2 cells in wells. Lens-free images before (A, B) and after (C, D) a 3 h exposure to NR. (A, C) Pinhole illumination resolves cell perimeters. PET films contain defects that appear as large spherical shadow (*arrows*). (B, D) Collimated light provides greater precision for quantifying the transmitted light by reducing the appearance of cell features and optical artifacts. The scale bar represents 100 µm. (E) Live absorbance measurements during cellular uptake of NR. Cells were categorized into two discreet morphologies: (*closed*) dense clusters, (*open*) spread monolayers. NR was present in the medium at 40 mg/L (l = 0.9 cm). The medium's absorbance contribution was removed via a blank. The error bars represent the standard deviation (n=10).

the appearance of artifacts (cf. Fig. 3A-D). A linear response to standard concentrations of aqueous NR was observed with collimated illumination filtered at 540 nm (Fig. S2 in Supporting Information). Thus, live quantitative imaging of the NRUA proceeded as tandem lens-free imaging with 1) a pinhole source to locate cells and, 2) a collimated source to quantify transmitted light.

As previously observed with HepG2 cells grown on a hybrid cell adhesion matrix (hCAM)[25] and fibrin[26] scaffolds, mature HepG2 cultures tend to form multicellular 3-D morphologies. Two distinct morphologies were observed for HepG2 cells grown in our hCAM-coated cell culture systems: 1) dense clusters (3-D), and 2) spread monolayers (2-D). Dense clusters of HepG2 cells appear slightly darker than the surrounding culture throughout the wide field-of-view of the lens-free image (Fig. S1E in Supporting Information). For image processing and data analysis, cultures were divided into two morphological subgroups (i.e., dense clusters and spread monolayers).

Neutral red uptake by living HepG2 cells is observable and quantifiable using lens-free imaging and tandem light sources. Live quantitative imaging was successful in the presence of aqueous NR. The cells gradually darken as they uptake NR (cf. Fig. 3B and 3D), with the dense clusters appearing darker than the spread monolayers. The absorbance of the accumulated neutral red is calculated from images collected with collimated light (Fig. 3E). Dense clusters and spread monolayers demonstrate a similar progression of NR uptake with a steady accumulation over the 3 h assay period. This trend is consistent with the standard protocol[10] and our reproductions of the standard protocol for similarly grown HepG2 cultures in 96-well plates (Fig. S3 in Supporting Information).

After live quantitative imaging, cell culture systems were subjected to the post-processing procedure of the standard protocol[15] (Fig. 4) to ensure the custom cell culture system did not influence the results. As expected, cultures treated with paraformaldehyde (dead cells) have no appreciable accumulation of NR as compared to the post-processing, while living cultures accumulated NR (Fig. 4A).

Live quantitative imaging detects the expected NR uptake by living cells of both morphologies (Fig. 4B). Paraformaldehyde-killed cells, however, have a slightly lower absorbance after NR incubation (Fig. 4B), which we attribute to a

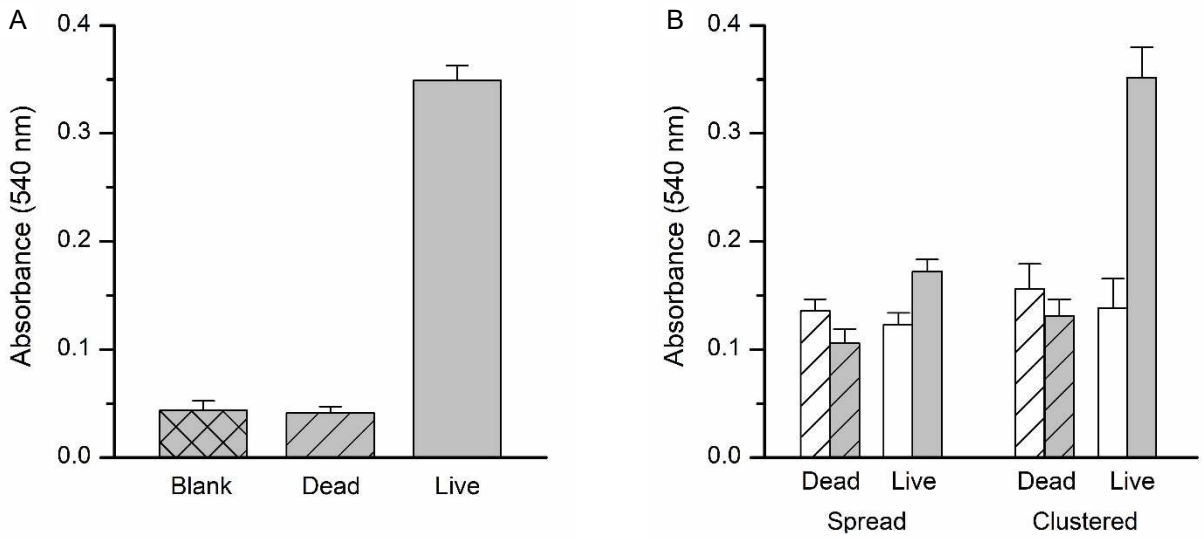

Figure 4. Comparison of the standard NRUA and live quantitative lens-free imaging with living and dead cultures. HepG2 cells were seeded on hCAM-coated lens-free culture systems and grown for 2 d past confluency. "Dead" cultures were killed with a 10 min exposure to 4% paraformaldehyde before staining to preserve cell structure. (A) Cell viability results using the standard NRUA. For direct comparison, dye extraction occurred after live quantitative lens-free imaging. NR absorbance was measured in the destain solution (50% ethanol, 1% acetic acid in DIW). The blank was an unseeded cell culture system. Error bars represent the standard deviation (n = 5). (B) Live quantitative lens-free measurements in dye-free medium before (white) and after (grey) the NR uptake. For quantification, cells were separated into two discreet morphologies: spread monolayers (Spread) and dense clusters (Clustered). Aqueous NR was present during imaging at 40 mg/L (l = 0.9 cm) for 2 h. Error bars represent the standard deviation (n=10).

destabilization of the fixed cell and release of cytoplasm during incubation at 37˚C. This effect further demonstrates how live quantitative imaging can yield more than standard staining protocol.

Although the trends of NR uptake were similar, light absorption by dense clusters and spread monolayers was drastically different. We explored this apparent disparity in NR uptake not as a metabolic distinction, but rather as a discrepancy in pathlength. If cells are nominally similar in volume and metabolic activity, the reduced cell area of dense clusters in a 2-D image may be an indicator of vertical growth. An increase in cell height leads to an increased sampling pathlength of the accumulated NR above each pixel, thus an increased absorbance in accordance with Beer's law. Previous work demonstrated that hCAM stimulates HepG2 cells to form regions of 3-D cultures.[25] Confocal microscopy of the live quantitative lens-free imaging cell cultures confirms the formation of 3-D structures on these hCAM surfaces (Fig. S4 in Supporting Information). The height of HepG2 monolayers ($\Delta A = 0.05 \pm 0.02$) vary from 4 μm to 6 μm, whereas cell clusters ($\Delta A = 0.24 \pm 0.04$) vary from 15 μm to 35 μm in height (Fig. S5 in Supporting Information). A disparity in NR uptake within a population presents a promising and inexpensive screening technique for estimating the height and progression of 3-D growth.

The extraction process for the standard NRUA presents a fundamental challenge for the confined environment of microfluidics. Live quantitative imaging alleviates this problem, while lens-free technology allows for integrating microscopic detection into microfluidic systems. Despite optical artifacts from non-uniformity of PDMS, the perimeters of individual cells are identified with pinhole illumination (Fig. 5). Spread cells exhibit a greater cell area while densely clustered cells (Fig. 5A oval) have a rounded body, which is consistent with early 3-D HepG2 cultures on peptide scaffolds[27]. NR accumulation is clearly apparent in collimated illumination (cf. Fig. 5B and 5D). NR absorbance is quantifiable at the cellular level by using the perimeter of individual cells as the region of interest for extracting the amount of transmitted light from collimated-light images (Fig. 5E). Densely-clustered cells exhibit a greater absorbance than spread cells, likely due to their transition to

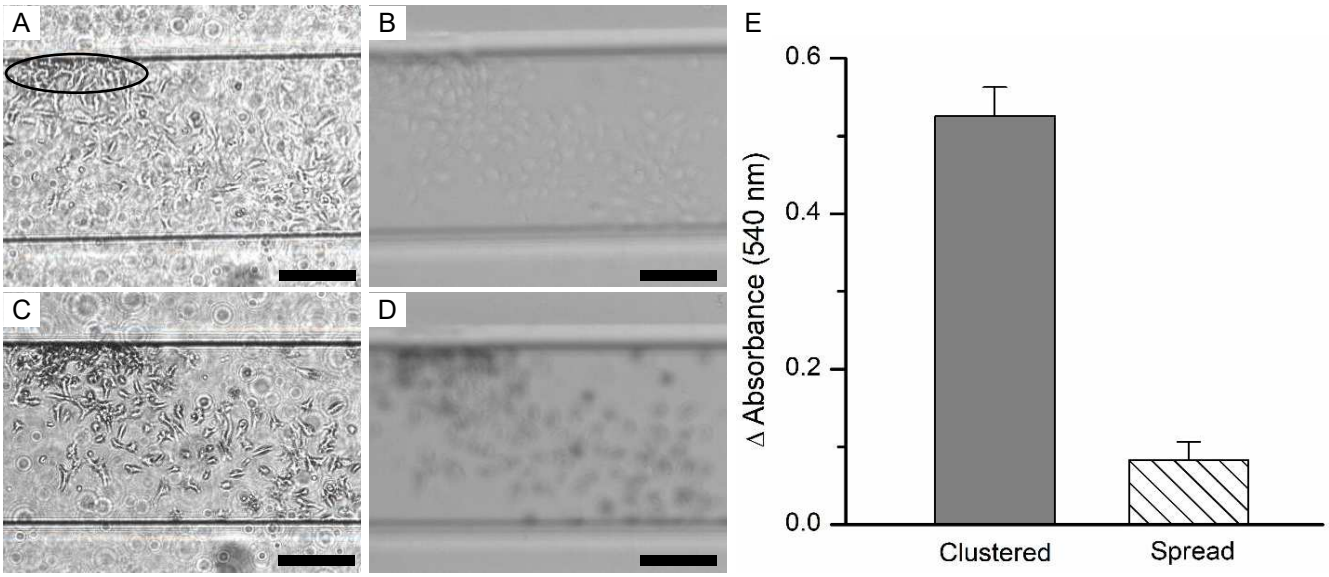

Figure 5. NR uptake by HepG2 cells within a microchannel. Lens-free imaging with (A,C) pinhole illumination and (B,D) collimated illumination before (A,B) and after(C,D) a 3 h exposure to NR. The oval in (A) identifies a dense cluster of cells. The scale bar represents 200 μm. (E) Quantified absorbance of neutral red within individual HepG2 cells. Cells were categorized into two discreet morphologies: (solid) dense clusters, (patterned) spread monolayer. The error bars represent the standard deviation (n=10).

vertical growth as described earlier. The NRUA and lens-free quantitative imaging provide an inexpensive and definitive means of identifying cell viability on a cell-by-cell basis for microfluidic applications. The minimal distance to sample (<150 μm) reduces the form factor of lens-free imagers allows for live quantitative imaging in microfluidics and lab-on-a-chip devices without the bulky housings of traditional microscopes.

Tandem lens-free imaging with pinhole and collimated light sources provides live quantitative imaging for the cellular uptake of a supravital stain. This approach reduces assay time by eliminating post processing, while expanding data collection to the sub-culture and cellular level. Delineation of the cell perimeter enables identification and subsequent quantification between morphological variants down to the cellular level, rather than an ensemble average of the entire culture that the standard protocols provide. Besides cell viability, NR uptake visually enhances cell morphologies exhibiting vertical growth, which presents an inexpensive screening method for 3-D culture growth. The reduced footprint of lens-free imaging enables high-throughput, *in-situ* screening of cell cultures for cell viability and 3-D growth at the point of use (e.g., incubator), where data is rarely collected due the inhibitory nature of integrating traditional microscopic and spectroscopic equipment.

EXPERIMENTAL SECTION

Cell culture systems for Lens-Free Imaging. Two different custom culturing systems were fabricated with a thin substrate (either <200 μm glass coverslips (#1) or 25 μm thick polyester films) to better resolve objects by minimizing the distance between the sample and imaging sensor. Polydimethylsiloxane (PDMS, Sylgard 184)[28] cell culture housings were fabricated in two formats: 1) well plates, and 2) microfluidic channels. PDMS was mixed at the suggested 10:1 mix ratio and cured at 70˚C for 8 h, unless specified otherwise.

Well-plate systems simulated tissue culture plates typically used in standard NRUA[15]. A hole punch fashioned a 7 mm diameter well in PDMS slabs. Two access ports are made with 4 mm diameter hole punch by carely cutting 1 mm into opposite sides of the 7 mm well. The result appears as three conjoined circles. The substrate and cover of the 7 mm diameter well were comprised of polyester (PET, 25 μm thick) or glass (#1 coverslip) that was sealed with a thin film of PDMS cured at room temperature for over 48 h.

Microfluidic systems were fabricated via soft lithography.[29] Briefly, a silicon wafer was

photolithographically micropatterned with SU-8. Reservoirs were fashioned with a 5 mm diameter hole punch. After cleaning with isopropanol, the PDMS was exposed to an oxygen plasma for 1 min, then covalently attached to #1 glass coverslips. Cell culture systems were sterilized with 70% ethanol. Flow in the microfluidic systems (400 μm wide, 70 μm high, 30 mm long) was controlled with gravity flow (Δh_{max} = 0.9 cm).

Cell Culture. Frozen stocks of human hepatoma cells (HepG2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS, 10% volume fraction), and incubated at 37˚C and 5% CO_2. Medium was changed every 24 h to 48 h. Upon reaching 60% to 80% confluency, cells were resuspended with 0.25% trypsin, diluted with DMEM containing FBS, and passed to a new tissue culture flask. Cells were passed at least once prior to seeding on the lens-free-imaging cell culture system.

Cell culture systems were coated with a hybrid cell adhesion matrix (hCAM) to promote cell attachment and 3-D growth. The first layer was added by filling the growth chamber with 400 μL of 50 mg/L fibronectin in Dulbecco's phosphate buffered saline (DPBS) for 1 h. The chamber was rinsed with DPBS and deionized water (DIW). The second layer was added by filling the chamber with 400 μL of 1 g/L poly(allylamine HCl) (PAH) in DIW for 1 h. The chamber was subsequently rinsed with DIW. All cell culture systems were filled with DPBS and incubated at 37˚C for at least 1 h prior to seeding cells.

When the cell culture reached 60% to 80% confluency, HepG2 culture were detached with 2 mL of 0.25% trypsin, which was quenched with 4 mL of DMEM with FBS. The cell suspension was adjusted to obtain a 60% to 80% confluency in the cell culture systems. Cell seeding occurred for up to 8 h before exchanging the cell suspension with growth medium. Growth medium was refreshed every 16 h to 24 h thereafter. Cultures in well-type culture systems grew to full confluency within 4 d, then continued for 2 additional days to develop 3-D growth. Cultures in microfluidic systems grew for 5 d to 6 d**.**

Lens-Free Imaging. The lens-free imaging system was equipped with an 8 megapixel (3264 x 2448 pixels) complementary metal–oxide semiconductor (CMOS) image sensor with a field-of-view of 10.14 mm^2 and a pixel spatial resolution of 1.12 μm (SOL Inc., KR). The custom optical microlayers on the CMOS image sensor minimize distortion of transmitted light by optimizing the incident angle for quantification. The imaging gap between the cell culturing system and the lens-free imaging sensor was 500 μm. The 25-μm pinhole LED light source (SOL Inc., KR) and collimated halogen light source (Zeiss) were filtered at a wavelength of 540 nm ± 35 nm bandwidth. Light sources were positioned 5 cm above the sample. Image J[30] was used to statistically analyze the pixel intensities from the digitized images.

Neutral Red Staining. We performed the NRUA similarly to the established protocol.[6] Negative controls were generated by killing cell cultures with a 10 min exposure to 4% paraformaldehyde to simulate “dead” cells while maintaining the cell body for live quantitative imaging. Growth medium was exchanged with DMEM with FBS and without phenol red (a spectroscopic interferent). After collecting pre-assay images, medium was replaced with 40 mg/L NR in DMEM with FBS. Imaging and medium refreshing occurred every hour. After up to 3 h, medium was replaced with the dye-free DMEM with FBS for collecting final images. Comparative optical images were collected with a Zeiss AxioCam MRc5 mounted on a Zeiss Axio Observer equipped with Zeiss Plan-Apochromat 10x/0.45 NA and 20x/0.8 NA phase contrast objectives.

Samples for confocal microscopy were rinsed with DPBS and fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 in DPBS, stained with 165 μM Alexa Fluor 546 Phalloidin in DPBS for 1 h, and rinsed with DPBS. Confocal images were collected with a Zeiss LSM 800 using a Zeiss Plan-Apochromat 40x/1.3 NA Oil objective and a 561 nm diode laser.

ImageJ[30] was used to manually analyze the NR uptake. HepG2 cells were categorized based on the density and shapes of cell bodies from

pinhole illuminated images. The "dense clusters" group consisted of 3 or more cells whose cell bodies appeared circular and did not exceed an area of 75 pixels (91 μm^2). Cells categorized as "spread monolayer" displayed an oblong or tubular morphology covering areas of up to 500 pixels (665 μm^2). For confluent cultures, areas of consistent cell morphology were identified on pinhole-illuminated images and marked as circular regions of 1976 pixels (2390 μm^2) (n=10 per morphology). For microfluidic cultures, regions of interest were confined to individual cells as defined by the saturated pixels from the pinhole image (n=10 per morphology). Marked regions were transferred to their respective collimated-light images to quantify the light intensity from samples and background (i.e., reference light intensity). The absorbance within regions was calculated with Beer-Lambert's law ($A = -\log i/i_o = \varepsilon lc$, where A = absorbance, i = light intensity through sample, i_o = reference light intensity, ε = molar extinction coefficient, l = pathlength, and c = concentration). The change in absorbance (ΔA) was defined as the difference in absorbance values obtained with dye-free medium before and after the NRUA and reported as the average ± the standard deviation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Image comparison of HepG2 cultures grown for 6 d on hCAM-coated coverslips, response of the lens-free imager to concentrations of neutral red in DPBS, plots of cytotoxicity testing of HepG2 cultures using the standard NRUA, confocal microscopy of a HepG2 culture grown on hCAM for 6 days, and quantification of NR uptake by HepG2 cultures prepared for confocal microscopy (PDF).

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Notes

The authors declare no competing financial interest.

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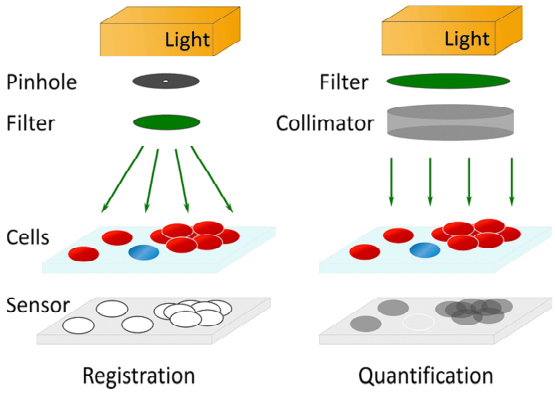
TOC Graphic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Quantification

Supporting Information

Lens-Free Imaging as a Sensor for Dynamic Cell Viability Detection Using the Neutral Red Uptake Assay

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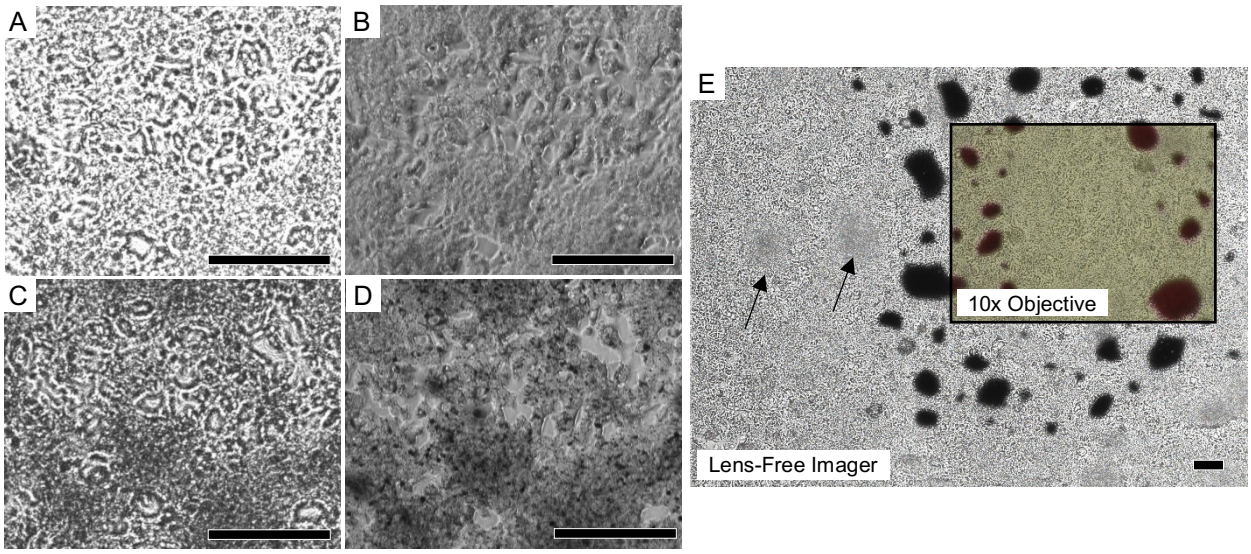

Figure S1. Image comparison of HepG2 cultures grown for 6 d on HCAM-coated coverslips. Aligned pinhole-illuminated lens-free imaging (A, C) and 10x phase-contrast optical microscopy (B, D) before (A, B) and after (C, D) a 2 h NR uptake assay. (E) Field of view of the lens-free image (whole) and respective 10x optical image (inset) before the NR assay. The arrows designate areas of dense clustering by the HepG2 cells. The black/red blotches are ink patterns used for image registration. Note, pinhole-illuminated images provide cell registration information for subsequent quantitative image analysis with collimator-illuminated images. The scale bars represent 200 μm.

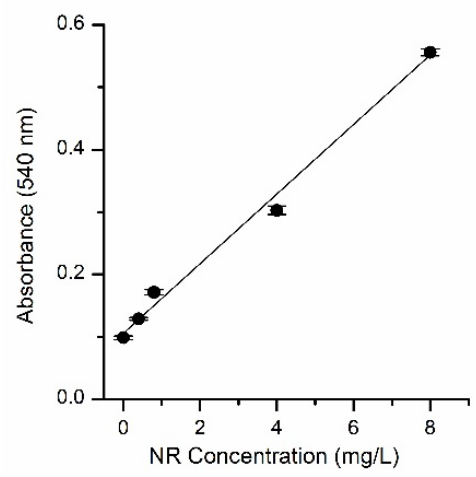

Figure S2. Response of the lens-free imager to concentrations of NR in DPBS. Collimated light was filtered at (540 ± 35) nm. Error bars represent the standard deviation (n = 5). Linear regression goodness of fit was $R^2 = 0.99$.

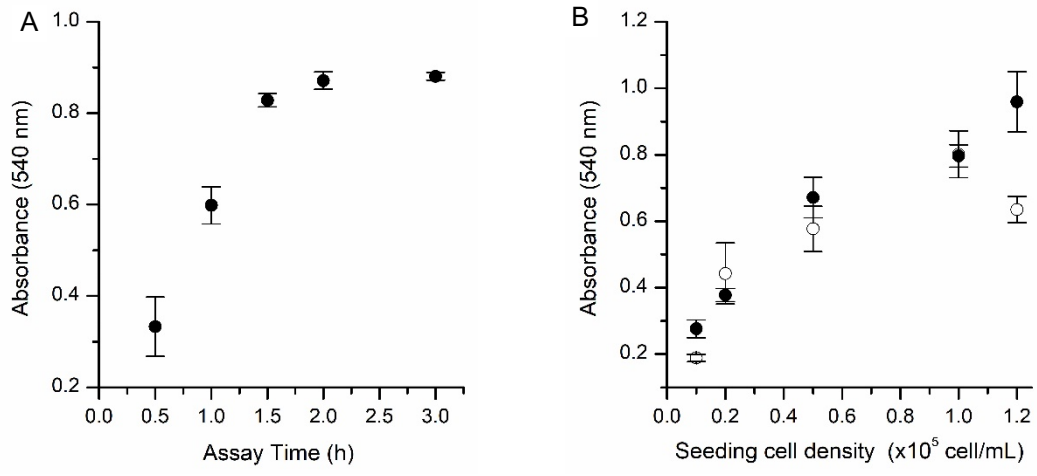

Figure S3. Cytotoxicity testing of HepG2 cultures using the standard NR protocol. (A) Impact of incubation time with aqueous NR. 96-well plates were treated with hCAM. Cell density: 1 x 10^5 cells/mL. The trend in absorbance as a function of NR-uptake duration is similar to the standard NRUA with 96-well plates[1]. (B) Impact of cell density at seeding. Cultures were grown in tissue-culture treated 96-well plates with hCAM (solid) and without hCAM (open) for 2 more days after reaching full confluency. Total culturing time was 6 d. NR incubation time was 2h. Error bars represent the standard deviation (n = 3).

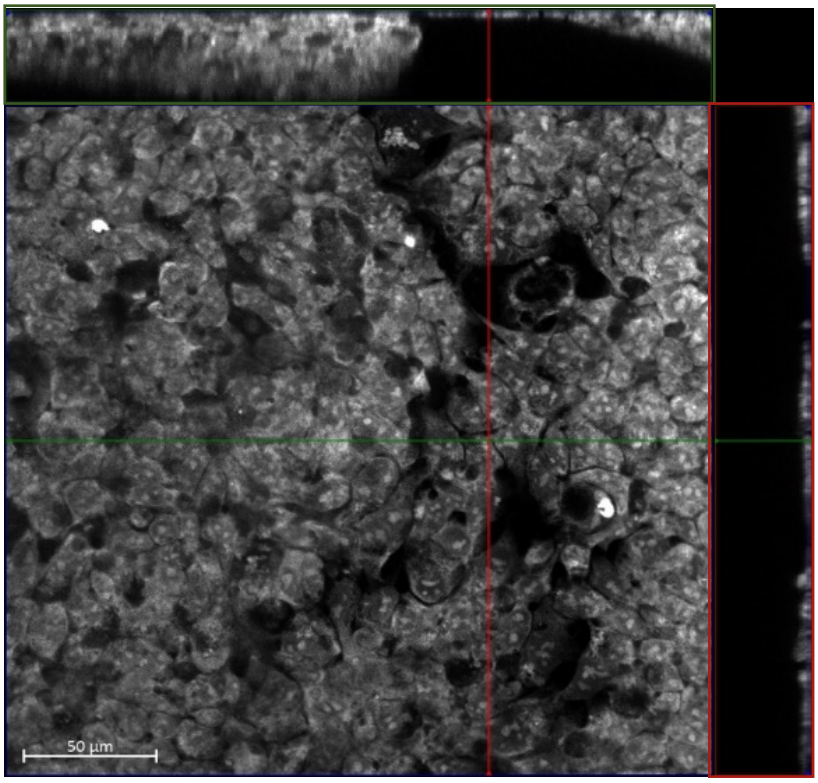

Figure S4. Confocal microscopy of a HepG2 culture grown on hCAM for 6 days (orthogonal view). The culture was fixed with 4% paraformaldehyde then stained with phalloidin Alexa 546. The *bottom left* displays the x-y cross section at Z = 1.5 μm above the glass surface. The *top* and *right* panels represent the z cross sections along the green and red line, respectively. The z-axis for the side panels progresses from the outside to inside. The scale bar applies to all panels.

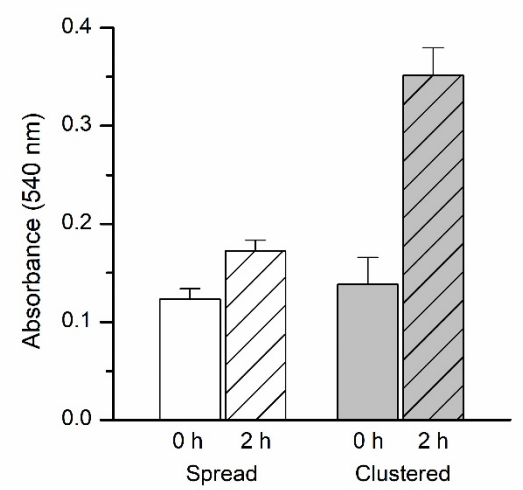

Figure S5. Quantification of NR uptake by HepG2 cultures prepared for confocal microscopy. Cells were seeded in well-type culture systems with hCAM-coated glass coverslips. NR uptake and lens-free imaging proceeded for 2 h. The absorbance of NR within HepG2 cells was determined with live quantitative image. Cells were categorized into two discreet morphologies: (*grey*) dense clusters, (*white*) spread monolayer. The error bars represent the standard deviation (n=10). Afterwards, cells were prepared for and imaged with confocal microscopy.

Reference

(1) Repetto, G.; del Peso, A.; Zurita, J. L. Neutral Red Uptake Assay for the Estimation of Cell Viability/Cytotoxicity. *Nat. Protoc.* **2008**, *3* (7), 1125-1131.