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LDL Cholesterol Uptake Assay Using Live Cell Imaging Analysis with Cell Health Monitoring

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Dr. Indrani Mukherjee 8/2/2018
Science Editor
JoVE

Dear Dr. Mukherjee:
We are pleased to submit the revised version of an original methods article entitled “LDL Cholesterol Uptake Assay Using Live Cell Imaging Analysis with Cell Health Monitoring” for your consideration for publication in JoVE. Our improved LDL uptake assay using live cell imaging provides a sensitive platform to serially quantify LDL uptake at different time points in various human cell lines. Concurrently with LDL uptake measurements, this technique allows for monitoring of the cell health and growth in real time; therefore, it could be a useful tool for monitoring potential cytotoxicity in studies screening for compounds regulating cholesterol metabolism. We have validated our method in three relevant cell lines including human hepatic carcinoma (HepG2) cells, human renal epithelial (HK2) cells, and human coronary artery endothelial cells (HCAECs), suggesting the broad applicability of the technique. Moreover, our findings support the validity and sensitivity of the LDL uptake quantifications as confirmed by the use of well-known LDL uptake inhibitors, Dynasore and PCSK9, as well as the LDL updake stimulator Simvastatin.

We believe this manuscript will be of useful impact to the field of cholesterol metabolism, and therefore is appropriate for publication in the JoVE. This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration!
Sincerely,

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TITLE:

LDL Cholesterol Uptake Assay Using Live Cell Imaging Analysis with Cell Health Monitoring

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KEYWORDS:

Low density lipoprotein, LDLR, LDL influx, LDL uptake, Dynasore, Simvastatin, recombinant PCSK9, dynamin, lipids, cholesterol, atherosclerosis.

SUMMARY:

This protocol provides an efficient approach to measuring LDL cholesterol uptake with real time influx rates using a live cell imaging system in various cell types. This technique provides a platform to screen the pharmacological activity of compounds affecting LDL influx while monitoring for cell morphology and hence potential cytotoxicity.

ABSTRACT:

The regulation of LDL cholesterol uptake through LDLR-mediated endocytosis is an important area of study in various major pathologies including metabolic disorder, cardiovascular disease, and kidney disease. Currently, there is no available method to assess LDL uptake while simultaneously monitoring for health of the cells. The current study presents a protocol, using a live cell imaging analysis system, to acquire serial measurements of LDL influx with concurrent monitoring for cell health. This novel technique is tested in three human cell lines (hepatic, renal tubular epithelial, and coronary artery endothelial cells) over a four-hour time course. Moreover, the sensitivity of this technique is validated with well-known LDL uptake inhibitors, Dynasore and recombinant PCSK9 protein, as well as by an LDL uptake promoter, Simvastatin. Taken together, this method provides a medium-to-high throughput platform for simultaneously screening pharmacological activity as well as monitoring of cell morphology, hence cytotoxicity of compounds regulating LDL influx. The analysis can be used with different imaging systems and analytical software.

INTRODUCTION:

The Low Density Lipoprotein Receptor (LDLR)-mediated LDL endocytosis is an important area of study since circulating LDL cholesterol levels are at the core of cardiovascular disease[1], kidney disease[2] as well as a variety of inflammatory diseases[3] and genetic disorders with mutations in cholesterol transport genes[4-7]. Studies in LDLR-mediated cholesterol influx have led to identification of multiple research tools, such as Dynamin inhibitors including the chemical Dynasore[8-10], as well as LDL-regulating proteins such as Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9)[11,12].

The LDL-LDLR endocytosis pathway begins with sequestering the LDL-LDLR complex on the cell surface into clathrin-coated pits[13]. Vesicles are then formed by invagination of the cell surface membrane internalizing the LDL-LDLR complex in vacuoles for transport inside the cell. As the formed vesicle matures into early and then late endosomes, the pH drops inside the late endosome, causing disassociation of the LDL from its receptor[14]. In the past, the methods of quantification of LDL influx depended on radio-labeled ^{125}I-LDL co-incubation with cells and subsequent extraction of the radio-labelled protein from cells for quantification[15]. This was then replaced by the use of fluorescently labelled LDL proteins such as DiI-LDL, and subsequent immunostaining or extraction of protein for fluorescent readings using a spectrophotometer or plate reader[15,16]. Fluorescently labelled LDL has also been used in Fluorescence-activated cell sorting (FACS) for analysis of internalization of LDL and cell surface LDL binding[17]. While these methods allow for collection of data after treatment, monitoring the viability of the cells during treatment is not possible.

The acidic pH in the late endosome allows the use of a pH-activated fluorescent LDL probe such

as pHrodo Red LDL that fluoresces after internalization[18,19]. This property allows for a continuous time course of LDL uptake assessment in live cells. Therefore, this protocol utilizes pHrodo Red-LDL fluorescence imaging in a live cell analysis to serially measure LDL uptake with concurrent monitoring for the cell health. The results indicate the reliability of this novel technique as tested over a four-hour time course in three different human cell lines, human hepatic carcinoma (HepG2) cells, human renal epithelial (HK2) cells and human coronary arterial endothelial cells (HCAEC). These cell lines are clinically significant to LDL clearance[20-27], kidney disease[28-31], and heart disease[32,33], respectively. In addition to monitoring the LDL influx, this protocol incorporates treatment with two well-known LDL uptake inhibitors, Dynasore Hydrate and recombinant PCSK9 protein as well as a statin inducer of LDLR expression and LDL uptake, simvastatin. Dynasore and recombinant PCSK9 each work through different pathways to reduce LDL uptake.

Dynasore is a small molecule inhibitor of Dynamins[10] and reduces LDL uptake by blocking clathrin-dependent endocytosis of LDL-LDLR complex[10,34]. Recombinant PCSK9, on the other hand, is a member of peptidase S8 family that binds to LDLR and inhibits its recycling to the cell surface after releasing LDL from the internalized complex by blocking required conformational changes[35,36]. Decreased cell surface LDLR density eventually leads to reduced LDL uptake by the cell. Statins, while directly blocking the 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase enzyme and thus cholesterol biosynthesis, are also known to upregulate the expression of LDLR[25,38] leading to increased LDL uptake. The sensitivity of this protocol is validated by detecting significant reductions in LDL influx in three clinically relevant human cell lines, HK2, HepG2 and HCAECs, by Dynasore and/or recombinant PCSK9, and a marked increase in LDL uptake in HepG2 cells by Simvastatin in a four-hour time course with monitoring for cell morphology/health. Taken together, this method provides a medium-to-high throughput platform for concurrently screening the pharmacological activity and cytotoxicity of compounds regulating LDL uptake in live cells.

PROTOCOL:

1. Seeding Cells in a 24-well Plate

1.1. Aspirate media off the cells, wash the cells with 5 mL of Dubelco's Phosphate Buffered Saline (dPBS), and aspirate the dPBS. For HepG2 cells in a 100 mm dish, use 1.5 mL of 0.25% Trypsin/EDTA, and for HK2 cells or HCAECs use 1.5 mL of 0.05% Trypsin/EDTA solution to detach the cells.

1.2. Incubate the plate in a 37 °C incubator for 4 minutes or until the cells are detached. Neutralize trypsin after a 4 minute incubation by adding 3 mL of complete media for HepG2 and HK2 or 3 mL of trypsin neutralizing solution, dPBS plus 5% fetal bovine serum (FBS), for HCAEC cells.

1.3. Transfer the cells into 15 mL conical tubes and centrifuge at 250 x g for 5 minutes, aspirate the media, and re-suspend the cell pellet in complete media.

1.4. Filter the cell suspension gently through a 40 µm mesh strainer to break up cell clumps. Do not wash the cells through the strainer.

1.5. Count the cells and plate them at an optimized density. For example, 5,000 cells per well of HepG2 cells or 10,000 cells per well of HK2 cells or HCAECs in a 24-well plate lead to optimal results.

1.6. Incubate the plate overnight at 37 °C to allow cells to attach.

1.7. The next day, change the cell media to base media for the cell line (without FBS) plus 5% Lipo-Protein Deficient Serum (LPDS) or low (2%) FBS media depending on the treatment (see 1.7). Then, continue incubation for 24 h to starve the cells. Use 500 µL of total media per well in a 24-well plate.

1.8. Treat the cells in one of three ways: Add 10 µg/mL of rPCSK9 (or vehicle) and return the cells to the 37 °C incubator for 1 hour, add 40 µM of Dynasore Hydrate (or vehicle, Dimethyl Sulfoxide) and return the cells to the 37 °C incubator for 10 minutes, or add 1 µM Simvastatin (or vehicle, Dimethyl Sulfoxide) and return the cells to the 37 °C incubator for 12, 18 or 24 hours. Use media with 5% LPDS for rPCSK9 or Dynasore treatments. Use low (2%) FBS media or media with 5% LPDS Simvastatin treatments.

Note: Treating the cells with desired compounds may be done at the time of media change for lipoprotein starvation (step 1.6) for long-term experiments, or prior to the analysis for short-term experiments. Alternatively, customized treatment times may be chosen based on the type and the purpose of the experiments.

1.9. Next, add 5 µL of pHrodo red-labelled LDL (1 mg/mL stock) to each well to obtain a final concentration of 10 µg/mL. Then, carefully remove any bubbles from the wells.

2. Live Cell Analysis

2.1. Immediately after adding the labelled LDL, place the plate in the live cell analysis system incubator (see **Table of Materials**) and allow the plate to equilibrate for 15 minutes to reduce condensation in the plate.

2.2. In the meantime, open the software and schedule the scan by adding the vessel holding the plate. Image 16 images per well at 1 hour intervals at 10X for 4 hours using the red and phase channels.

2.3. Create a Plate Map to use for data processing.

2.3.1. Click on the **Properties** tab. Choose the **Plate Map**. Input the cell type and treatments in **Compounds** tab.

2.3.2. Click on the **Regions** tab, select each set of replicates, and save as regions.

3. Set up Analysis Parameters

3.1. Once an experimental run is complete, create an Image Set in the software to train the computer to quantify each parameter included in the counting set.

3.1.1. In the software, open the plate view, then in the **Analysis Job Utilities** box choose **Create or Add to Image Collection**.

3.1.2. Select **New Image Collection**, type a name for the image collection, and choose the **Red** and **Phase** channels by checking the boxes next to the channels.

3.1.3. Select 5 more images and add to image collection by adding to the current image collection.

3.2. Create a Processing Definition for the cells. **Table 1** includes the parameters for the HepG2, HK2, and HCAE cell processing definitions for this LDL influx protocol.

3.2.1. In the **Analysis Job Utilities** box, choose **New Processing Definition**. Choose the Image Collection named in step 2.1.2 from the drop down menu. Input the parameters for the type of cells from **Table 1.**

3.2.2. In the **Preview box**, use the drop down menu to select **Preview All.**

3.2.3. In the **Analysis Mask** box, check the **Confluence Mask** and the **Red Object Mask** boxes to view the area included in the analysis for the processing definition. See **Figure 1**.

3.2.4. Scroll through the image collection to ensure the cells and LDL are included in the mask. Select **File** and **Save** the Processing Definition.

3.3. Analyze the set of images from the experimental run.

3.3.1. Open the experiment in plate view. In the **Analysis Job Utilities** box, choose **Launch New Analysis Job**. Select the saved Processing Definition.

3.3.2. Name the analysis job, choose the time range for analysis, and highlight the experimental wells to analyze. Click the **Launch** button.

4. Analysis and Data Processing

4.1. Once the Analysis Job is complete, export the data:

4.1.1. Select the completed analysis and press **View.** In the **Utilities** menu, choose **Metric/Graph Export.**

4.1.2. In the *Regions* menu, choose **All Wells**, and in the *Group* menu, to obtain the mean values for each set of wells as a group choose **Replicates** and for exporting the individual values for each well choose **None**.

4.1.3. In the **Red Object metric** menu, choose the **Total Red Object Integrated Intensity (RCU x µm²/image)**. This parameter indicates the sum of the red signal intensity (in RCU) times the area of the red signal (in µm²) in all the images across each well, which corresponds to the total LDL uptake by the cells.

4.1.4. Click the **Data Export** button. Check **Break data down into individual images**. The data is automatically copied on a clipboard and can be pasted to a new spreadsheet file.

4.1.5. In the **Phase Object metric** menu, choose the **Confluence (Percent)**. Check **Break data down into individual images**. Click the **Data Export** button. The data is automatically copied on a clipboard and can be copied to the spreadsheet file containing Total Red Object Integrated Intensity data.

4.1.6. Apply the conversion of the percent confluence to total area with the following equation.

Total Phase Area (µm²/image) = Confluence (%) × (Image Height (Pixels) × Resolution) × (Image Width (Pixels) × Resolution)

Note: The confluence (%) parameter indicates the percent confluence of the phase area per image that corresponds to the area of the cells in each well. This metric should be converted into total phase area for each individual image once exported. The image specifications for phase channel (image height, width and resolution) to be used with the above formula for each experimental vessel can be found by referring to **Vessel Properties** under **Image Channels.**

4.1.7. Normalize Total Red Object Integrated Intensity to the Total Phase Area as calculated in 4.1.6 using the following formula for each individual image to eliminate the variability in the cell density across the wells.

4.1.7.1. Divide the **Total Red Object Integrated Intensity (RCU x µm²/image)** values of each image by its corresponding **Total Phase Area (µm²/image)** to obtain **LDL Uptake (RCU)** values per image.

4.1.7.2. Then, average the **LDL Uptake (RCU)** data of all images of each well to obtain the average LDL uptake in each well and then average the LDL uptake values of all the replicate wells to obtain group means. These data are the final LDL uptake values and may be used for illustration and statistical analysis using the software of choice.

REPRESENTATIVE RESULTS:

Live Cell Imaging Allows for Reliable Monitoring of Cell Health During Cholesterol Influx Studies in Three Human Cell Lines

We validated our assay in three human cell lines in which cholesterol homeostasis regulation plays a major pathophysiological role, including human hepatic carcinoma (HepG2) cells, human renal epithelial (HK2) cells, and human coronary artery endothelial cells (HCAECs). We used a live cell imaging system to perform the LDL uptake assay in a 4-hour time course with serial

measurements at 1-hour intervals. Our results indicate that all the cell types tested were compatible with this new technique and result in curves indicating continuous LDL uptake for the duration of the influx study with 4.33 hours as the final endpoint (**Figure 2**). The influx data shown in **Figure 2** were obtained by normalizing the total pHrodo Red-labeled LDL fluorescence (total red object integrated intensity per image in RCU x µm2/image) to the total cell area in each image of each well (phase object area per image in μm^2/image) to eliminate the variability in the cell density across the wells. Furthermore, to validate the sensitivity of the LDL influx assay for the purpose of screening compounds affecting LDL cholesterol uptake, we used two positive controls known to inhibit LDL influx, Dynasore and rPCSK9, and one positive control known to induce LDL uptake, Simvastatin. As validated by our results, following treatment with Dynasore and rPCSK9, all three tested human cell lines (HepG2, HK2 and HCAEC) showed significant reductions in LDL influx over a 4-hour time course (**Figure 2A-C**). For example, **Figure 2A** shows that the LDL influx is reduced in HepG2 cells with treatment of Dynasore over the time course, compared to the cells treated with DMSO; while the DMSO as the vehicle control for Dynasore control had no significant effect on LDL influx compared to the untreated control group. Furthermore, our findings showed a marked increase in LDL uptake by HepG2 cells following treatment with Simvastatin (**Figure 3**), supporting the sensitivity of this method to detect significant alterations in the LDL influx. At about the 4.5 hour time point, which is a typical time point in LDL uptake studies, the LDL influx is significantly reduced with either Dynasore or rPCSK9 treatment, and increased by Simvastatin treatment (**Figure 4**).

A major advantage to using live cell imaging for LDL uptake studies is that this system provides real time images of the cells in each well that could be used to monitor potential cytotoxicity of the examined compounds. **Figures 5-7** illustrate representative images of the three cell lines investigated at the initial time point (0.33 h) and final endpoint (4.33 h) as a visual reference for the net LDL influx. The images confirm the normal morphology of the cells following Dynasore or rPCSK9 treatments, indicating both effectiveness and safety of these compounds.

Live Cell Analysis Gives Reliable Serial Quantitative Cholesterol Influx Measurements with Various Treatments

A major advantage of using this protocol with a live cell imaging system is the ability to collect data throughout the time course and compare LDL influx at multiple time points rather than just one final time point as traditionally done. Using this protocol, we are able to calculate the percent reduction in LDL influx at the terminal time point as well as at 1 hour intervals throughout the time course. **Table 2** summarizes the reduction in LDL influx in three tested human cell lines following 10 min pre-treatment with 40 µM Dynasore or 1 hour pre-treatment with 10 µg/mL rPCSK9. At 4.33 hours as the final study end point, treatment with Dynasore at 40 µM significantly reduced LDL influx in HepG2 cells, HK2 cells and HCAECs by 53%, 68% and 54%, respectively **(Table 2A-C)** and treatment with rPCSK9 at 10 µg/mL resulted in 55% reduction in LDL influx in HK2 cells **(Table 2B)**. In addition to quantifying the terminal time point as in traditional assays, we are able to perform a quantitative analysis in the reduction of LDL influx due to treatment at each time point of the experiment. For example, **Table 2B** shows that treatment with rPCSK9 in HK2 cells resulted in a reduction of LDL uptake by 79% at 1.33 hours, 67% at 2.33 hours, 59% at

3.33 hours post treatment compared to untreated cells. This protocol provides a reliable method for quantitative analysis of LDL influx after treatment.

FIGURE AND TABLE LEGENDS:

Figure 1. Processing Definition Mask. Representative images of HK2 cells are depicted following the application of the appropriate processing definition (detailed in **Table 1**). Shown are HK2 cells without masking **(A)**, with Phase Mask applied **(B)**, with Red Object Mask applied **(C)**, or with both Phase and Red Object masks applied **(D)**. Scale bar = 100 μm.

Figure 2. Reduction in LDL uptake using live cell imaging system over a 4.33 h time course. Live cell analysis system is used to measure LDL influx in human hepatic carcinoma (HEPG2) cells (**A**), human renal tubular epithelial (HK2) cells (**B**), and human coronary artery endothelial (HCAE) cells (**C**). The cells were treated with Dynasore (10 minutes before the run) or rPCSK9 (one hour before the run) as positive controls. DMSO was used as a vehicle for Dynasore treatments. Positive controls significantly decreased the LDL influx in all 3 cell lines. LDL influx values were obtained by normalizing the total red object integrated intensity (RCUxμm^2/image) to the total phase object area (μm^2/image). Data are mean±SEM. N = 6 wells/group. Data are representative of 2 or 3 independent experiments. ****p < 0.0001 vs blank, and ####p < 0.0001 vs DMSO, using two-way ANOVA.

Figure 3. Increase in LDL uptake using live cell imaging system over a 4.33h time course. Live cell analysis system is used to measure LDL influx in human hepatic carcinoma (HEPG2) cells. LDL uptake is significantly increased following treatment with Simvastatin for 12 hours (**A**), 18 hours (**B**), or 24 hours (**C**) using media containing 2% FBS. The 24 hour time point was also performed with media containing 5% LPDS (without FBS). DMSO was used as negative control. LDL influx values were obtained by normalizing the total red object integrated intensity (RCU x μm^2/image) to the total phase object area (μm^2/image). Data are mean±SEM. N = 6 wells/group. Data are representative of one independent experiment. ****$\mathbf{p}$ < 0.0001 vs DMSO, using Student's t-test.

Figure 4. Significant LDL influx reduction by LDL uptake-lowering agents at 4.3 hour time point. LDL influx is significantly reduced in human hepatic carcinoma (HepG2) cells (**A**), human renal tubular epithelial (HK2) cells (**B**), and human coronary artery endothelial (HCAE) cells (**C**) following treatment with LDL uptake inhibitors Dynasore for 10min or rPCSK9 for 1 hour. LDL influx is markedly increased by Simvastatin in HepG2 cells after 12, 18 or 24 hours treatments (**D**). Data are mean±SEM. N = 6 wells/group. Data are representative of 2 or 3 independent experiments. ****p < 0.0001 using two-way ANOVA.

Figure 5. Reduced LDL influx in hepatocellular carcinoma (HepG2) cells by LDL uptake-lowering agent, Dynasore. Representative images of the phase object and red object for HepG2 cells are depicted at 0.33 hour (left panels) and the 4.33 hour endpoint (right panels) show healthy status of cells. 40 μM Dynasore, known to reduce LDL-cholesterol uptake, was used as positive control (**C**). Images were taken at 10X magnification. Scale bar = 100 μm.

Figure 6. Reduced LDL influx in human renal tubular epithelial (HK2) cells by LDL uptake-lowering agents, Dynasore and rPCSK9.
Representative images of the phase object and red object for HK2 cells depicted at 0.33 hour (left panels) and the 4.33 hour endpoint (right panels) show healthy status of the cells. 40 µM Dynasore (**C**), or 10 µg/mL rPCSK9 (**D**), known to reduce LDL cholesterol uptake, were used as positive control. Images are taken at 10X magnification. Scale bar = 100 µm.

Figure 7. Reduced LDL influx in human coronary artery endothelial cells (HCAECs) by LDL uptake-lowering agent, Dynasore.
Representative images of the phase object and red object for HCAECs depicted at 0.33 hour (left panels) and the 4.33 hour endpoint (right panels) show healthy status of the cells. 40 µM Dynasore, known to reduce LDL-cholesterol uptake, was used as positive control (**C**). Images were taken at 10X magnification. Scale bar = 100 µm. Data are mean±SEM. N = 6 wells/group. Data are representative of 2 or 3 independent experiments.

Table 1. Processing Definition Parameters. These parameters are specific for the analysis system used in this protocol. Parameters should be set up to analyze red area in the red channel and the area of the cell in the phase channel. Parameter settings for HepG2, HK2, HCAE cell lines are presented.

Table 2: Percent Change in LDL influx in HepG2, HK2 and HCAE cells treated with Dynasore, rPCSK9, or Simvastatin at 4.3 hours. A. HepG2 cells **B**. HK2 cells **C**. HCAE cells **D**. HepG2 cells.

DISCUSSION.

In the current protocol, we demonstrate the utilization of live cell imaging as a new and more effective method for measuring real time LDL uptake over a time course in various human cell lines. Human hepatic carcinoma (HepG2) cells are commonly used in studies screening for cholesterol-lowering therapeutics[21-26,39,40]. Therefore, we chose this cell type for testing the capability of a live cell imaging system for LDL influx studies. Our results indicate that HepG2 cells are compatible with this new technique and result in a sigmoid-like curve indicating continuous LDL uptake for the duration of the influx assay until 4.33 hours as the final endpoint (**Figures 2A and 3**).

Cholesterol homeostasis plays a major role in the pathophysiology of various nephropathies. Indeed, cholesterol accumulation in renal tissue is a major contributor to renal fibrosis leading to chronic kidney disease and is a major pathology in various nephropathies[28-31]. Hence, we examined our method in human renal epithelial (HK2) cells as a popular and reliable cell line utilized in the field of nephrology. Our data also supported the feasibility of the live cell imaging system to measure LDL influx in HK2 cells. As shown in **Figure 2B**, HK2 cells took up LDL cholesterol linearly throughout the duration of the influx study (4 hours).

Due to the importance of the cholesterol metabolism in the development and progression of atherosclerosis[32,33,41], the leading cause of cardiovascular disease, which in turn is the number-

one killer worldwide[42], we aimed to validate our method in an atherosclerosis-relevant cell type. We used Human Coronary Arterial Endothelial Cells (HCAECs), as these are one of the first cell types to be exposed to a cholesterol insult in the coronary artery lumen of an atherosclerosis patient. Our data shown in **Figure 2C** indicates that this LDL influx method also works effectively with HCAECs. The resultant graph is a sigmoid-like curve similar to that of HepG2 cells.

To test the validity and sensitivity of this improved LDL influx assay for screening compounds affecting LDL-cholesterol uptake, we used three controls, LDL uptake-lowering agents Dynasore and rPCSK9, and LDL influx activator Simvastatin. Here, we treated the above mentioned cell lines (HepG2, HK2 and HCAECs) with optimized concentrations of Dynasore or rPCSK9 prior to the influx assay. Our results showed that all three tested cell lines responded to the treatments with significant reductions in LDL influx over a 4-hour time course (**Figure 2**). For instance, at 4.33 hours as the final time point, treatment with Dynasore at 40 μM significantly reduced LDL influx in HepG2 cells, HK2 cells and HCAECs by 53%, 68% and 54%, respectively ($p<0.0001$; **Figure 2A-C** and **Table 2A-C**). In addition, rPCSK9 at 10 μg/mL caused a marked 55% reduction in LDL influx in HK2 cells ($p<0.0001$; **Figure 2B** and **Table 2B**). Moreover, our findings showed that treating HepG2 cells with Simvastatin resulted in a marked increase in LDL uptake (**Figure 3**), supporting the sensitivity of this method to detect significant alterations in the LDL influx. Studies of treatment with rPCSK9 in HepG2 and HCAEC cells are not included in this protocol as rPCSK9 is used as an additional control treatment with well-documented results, but is costly to buy in small quantities. Therefore rPCSK9 was only used to validate this protocol in HK2 cells.

Live cell imaging analysis, along with the functional and timely measurement of LDL influx, allowed for continuous monitoring of the health and morphology of the cells. This advantage can efficiently detect potential cytotoxicity of the applied compounds, making this method an ideal technique for simultaneously monitoring pharmacological activity and cytotoxicity. **Figures 5-7** illustrate representative images of the three tested cell lines at the final endpoint (4.33 h) as a visual reference for the effect of the treatments on net LDL influx and also shows the healthy morphology of the cells following the tested treatments. We recommend visual inspection of all the images from each well to assure the heathy morphology of the cells for the duration of the study. For example, in data not shown, when images of HepG2 cells treated with 80 μM Dynasore were inspected, we observed indications of cell detachment as the edges of the cells appeared to be lifting off the plate, suggesting cell detachment at higher concentrations of Dynasore. Furthermore, high concentrations of simvastatin (3-10 μM) also led to altered morphology indicating induced apoptosis as reported for high dosages of statins[45]. This protocol was used to perform a titration of the Dynasore treatment at 20-80 μM, and Simvastatin at 0.5-10 μM concentration, following which the cell images were used to analyze the health of the cells and determine potential ctotoxicity of the treatments at various concentrations. Results suggested the use of 40 μM for Dyansore and 1 μM for simvastatin as optimal concentrations.

Lastly, we suggest performing a cell density titration study if another cell line is to be tested with this method in order to identify the optimal cell number per well to obtain consistent results. Our cell density optimization study showed that 10,000 cells/well in a 24-well plate lead to consistent LDL influx outcomes for HK2 and HCAE cells. It is important to note that for this LDL influx assay

using live cell imaging system, a monolayer of non-confluent cells distributed evenly on the wells is desirable as clumps of cells may give rise to errors in the final normalized LDL influx values. The reason for this is that for the normalization of the influx data, the phase object area is used as a measure of cell density and this parameter can be adversely affected when cell clumps are formed. We observed that HepG2 cells have a tendency to form clumps when seeded at densities higher than 5,000 cells/well causing inconsistent influx results; therefore, we used 5,000 cells per well in a 24-well plate as optimal density for HepG2 cells.

Collectively, our method provides a medium-to-high throughput platform for screening the pharmacological activity and cytotoxicity of compounds regulating LDL influx concurrently. This method can be readily adapted for use with other fluorescently-labeled ligands that enter the lysosomal compartment to evaluate ligand uptake in real time. While this protocol offers specifications for InCucyte live imaging and analysis system, the protocol can be adapted for alternative imaging systems such as Cellomics.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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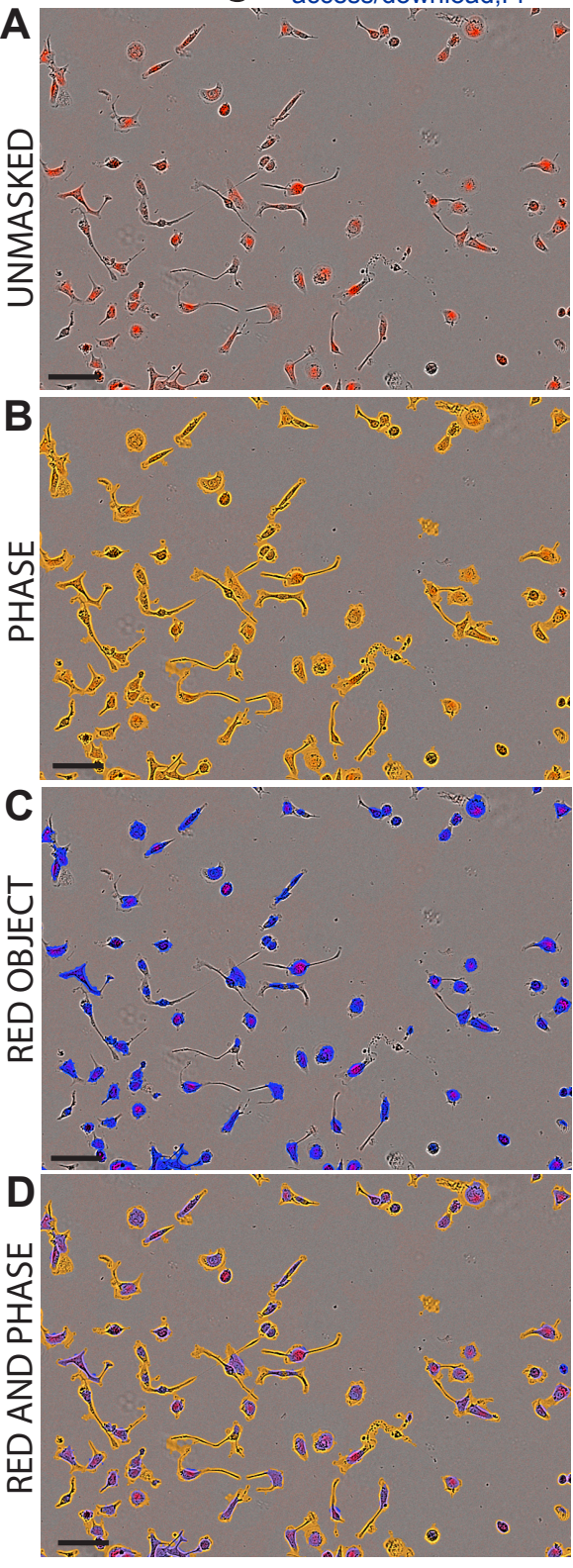

Figure 2

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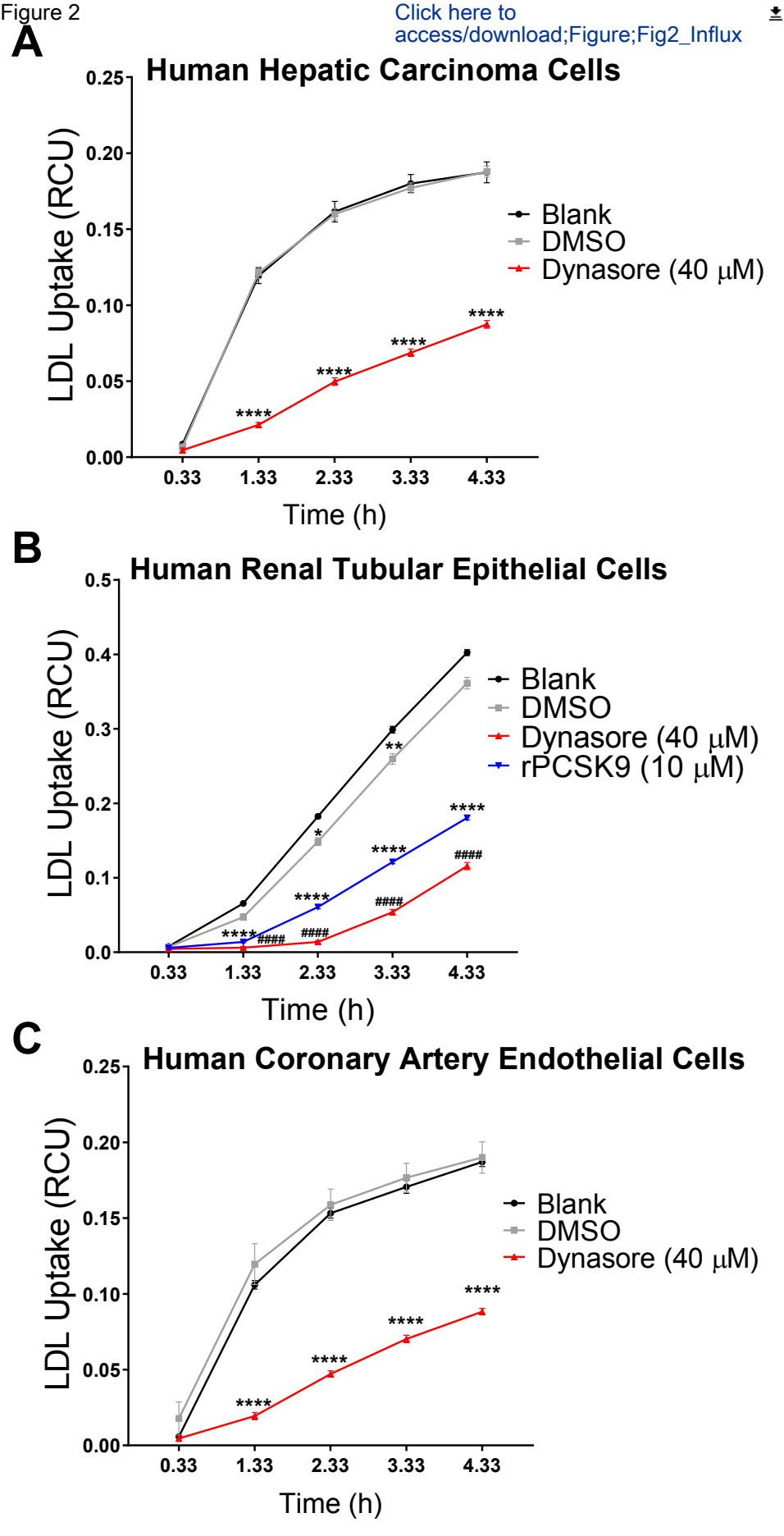

Figure 3

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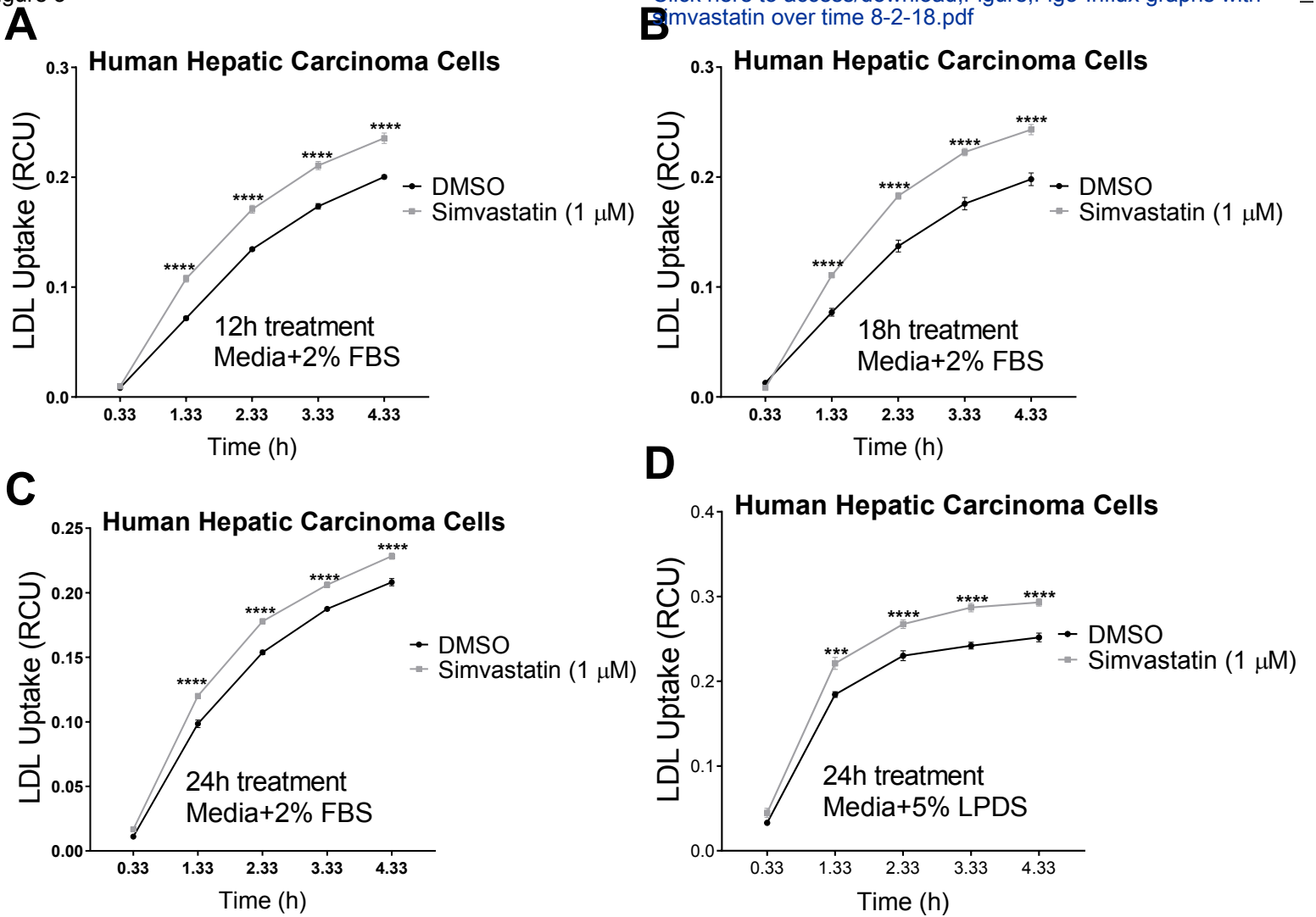

Figure 4

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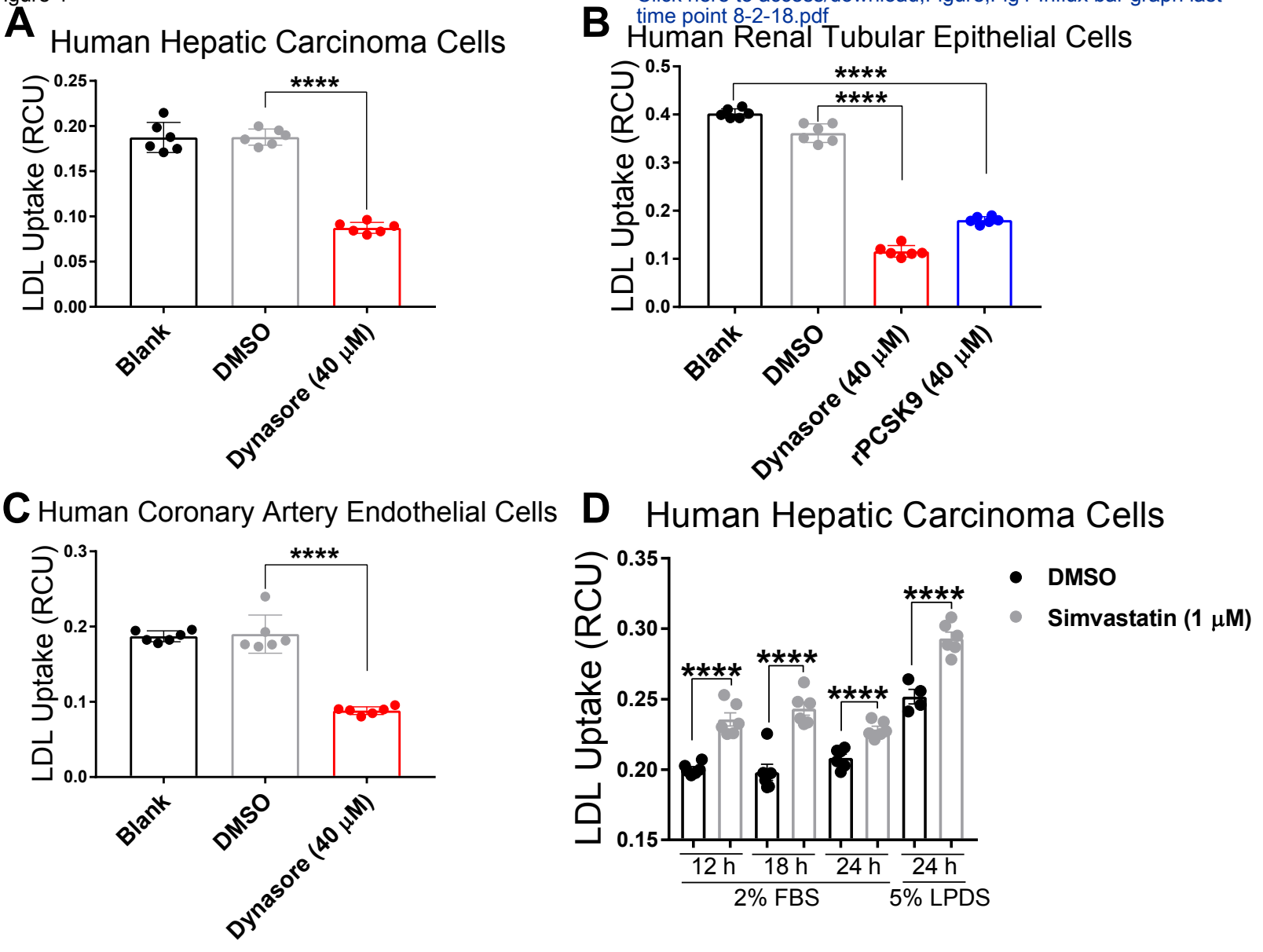

Figure 5

Human Hepatic Carcinoma Cells

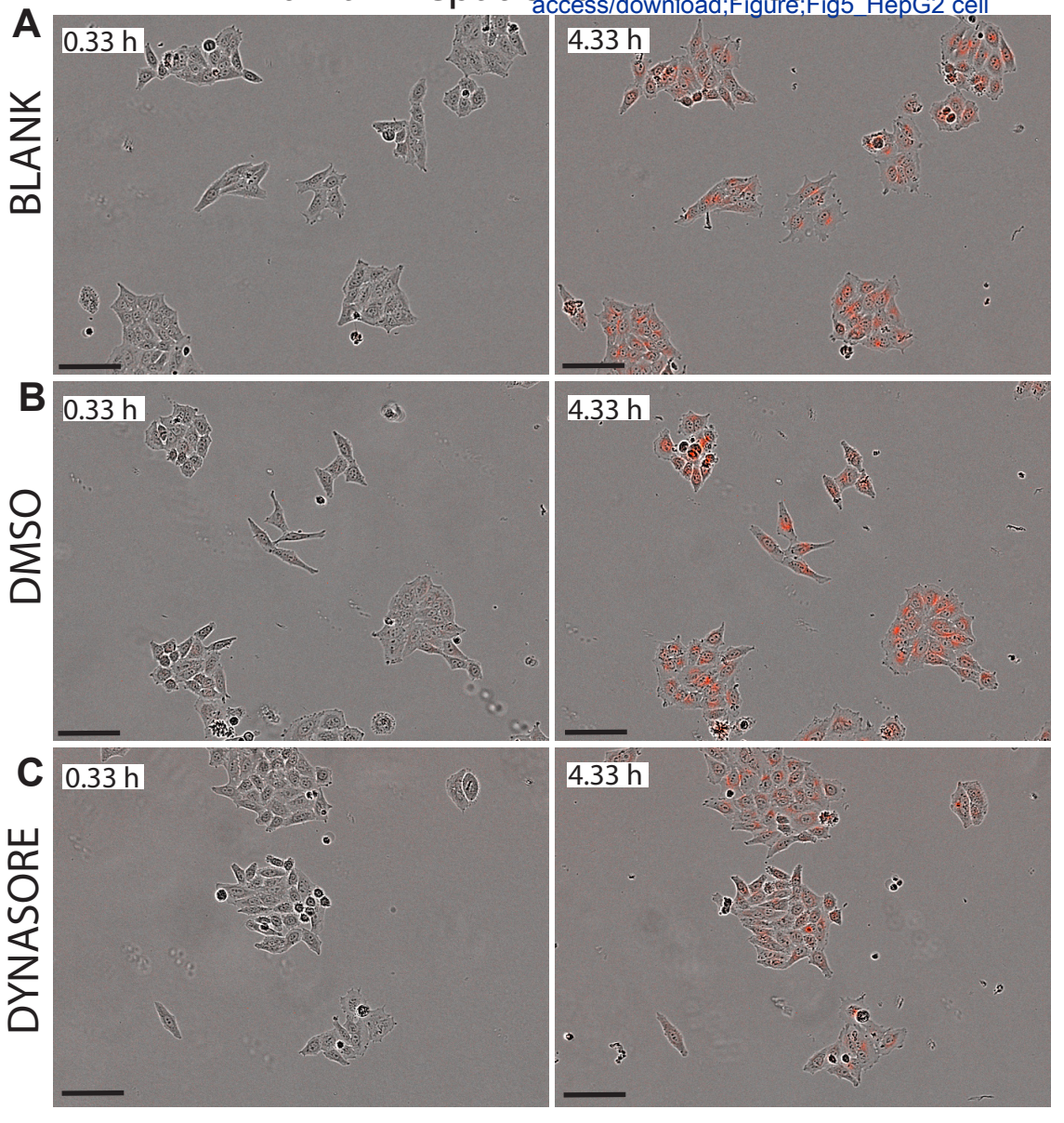

Figure 6

Human Renal Tubular Epithelial Cells

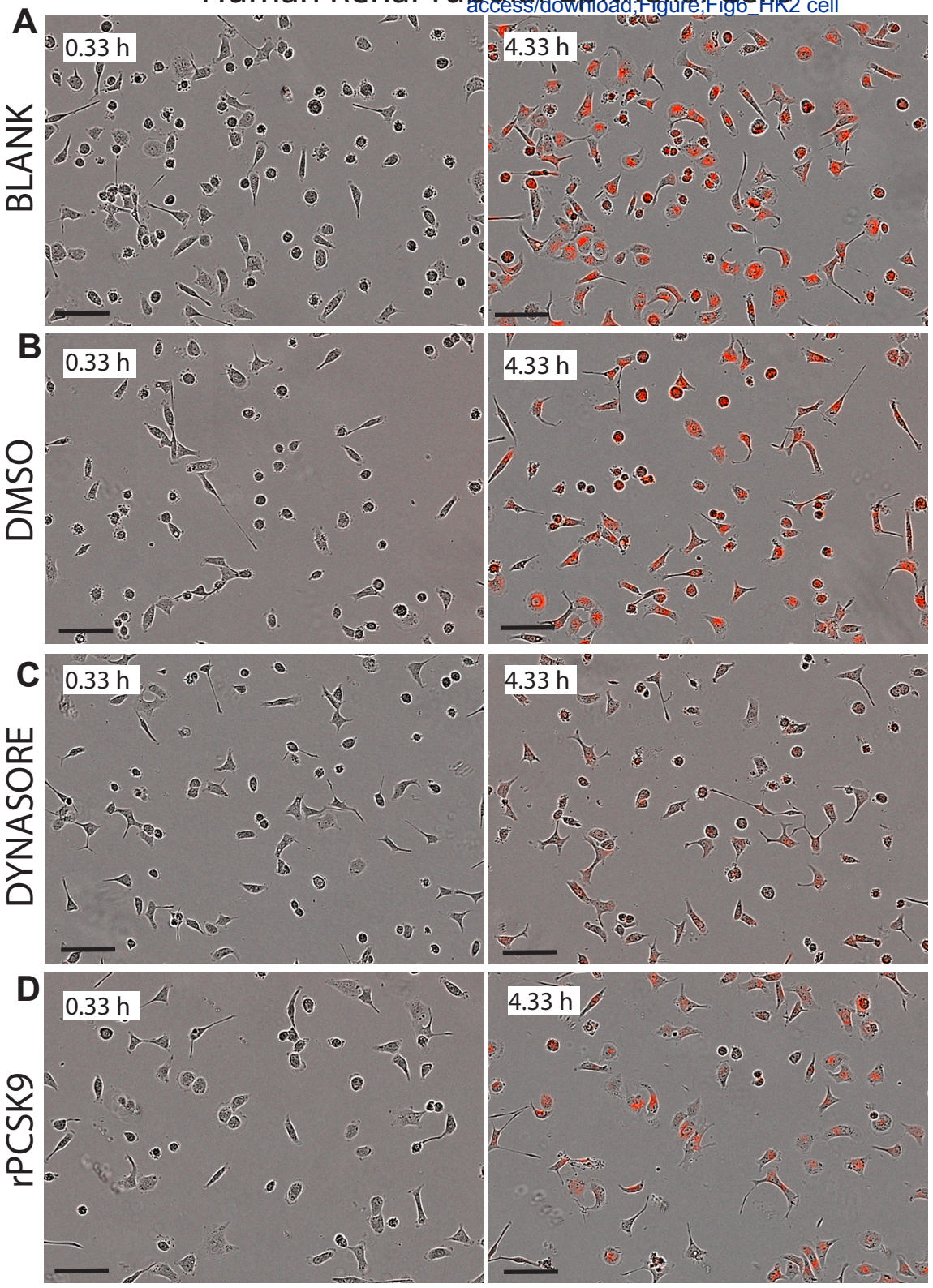

Figure 7

Human Coronary Artery Endothelial Cells

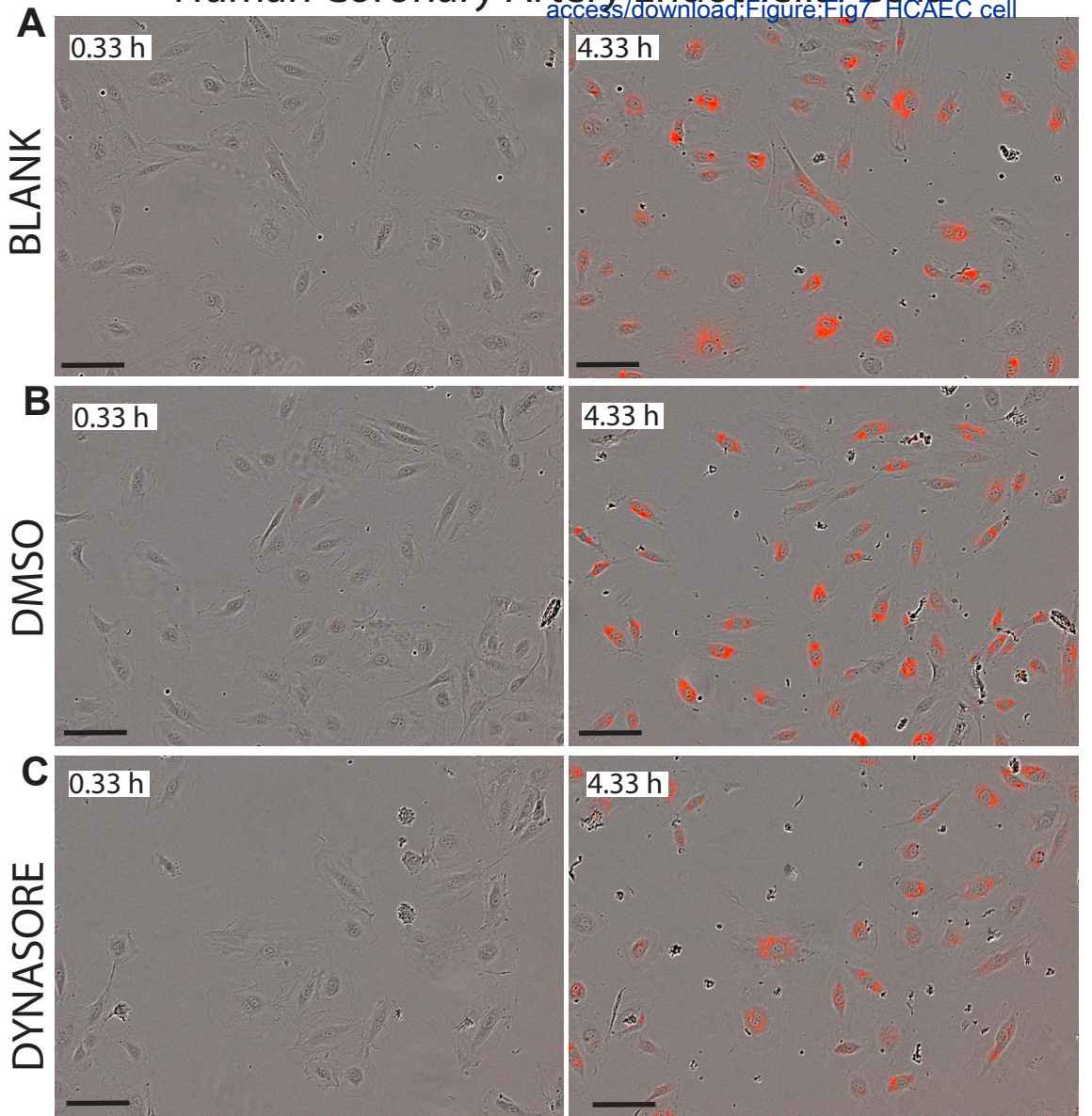

Table 2 Click here to access/download;Table;Table 2 8-2-18.xlsx

A. HepG2 Cells

Elapsed Time (h)	HepG2 + DMSO	HepG2 + Dynasore
1.33	2%	-83%
2.33	-1%	-69%
3.33	-2%	-61%
4.33	0%	-53%

B. HK2 Cells

Elapsed Time (h)	HK2 + DMSO	HK2 + Dynasore	HK2 + PCSK9
1.33	-28%	-87%	-79%
2.33	-19%	-91%	-67%
3.33	-13%	-79%	-59%
4.33	-10	-68%	-55%

C. HCAE Cells

Elapsed Time (h)	HCAEC + DMSO	HCAEC + Dynasore
1.33	13%	-84%
2.33	4%	-70%
3.33	4%	-60%
4.33	2%	-54%

D. HepG2 Cells

Elapsed Time (h)	HepG2 + FBS + Simvastatin 12 h	HepG2 + FBS + Simvastatin 18 h	HepG2 + FBS + Simvastatin 24 h	HepG2 + LPDS + Simvastatin 24 h
1.33	50%	44%	22%	20%
2.33	27%	33%	16%	16%
3.33	21%	27%	10%	19%
4.33	18%	23%	10%	16%

Table 1

HepG2	HK2	HCAEC
Phase (Analyze) ➢ *Parameters:* ○ Segmentation adjustment: **0.6** ➢ *Cleanup:* ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**1300.0**, ☐max=**1300.0** ○ Eccentricity: **not selected**	**Phase (Analyze)** ➢ *Parameters:* ○ Segmentation adjustment: **0.6** ➢ *Cleanup:* ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**600.0**, ☐max=**600.0** ○ Eccentricity: **not selected**	**Phase (Analyze)** ➢ *Parameters:* ○ Segmentation adjustment: **0.6** ➢ *Cleanup:* ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**400.0**,☐max=**400.0** ○ Eccentricity: **not selected**
Green (Not Selected)	**Green (Not Selected)**	**Green (Not Selected)**
Red (Analyze) ➢ *Parameters:* ○ **Top-Hat** ○ Radius(um): **50** ○ Threshold (RCU): **0.3** ○ Edge Split **Off** ➢ *Cleanup* : ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**96.0**, ☐max=**96.0** ○ Eccentricity: **not selected** ○ Mean Intensity: **not selected** ○ Integrated Intensity: **not selected**	**Red (Analyze)** ➢ *Parameters:* ○ **Top-Hat** ○ Radius(um): **50** ○ Threshold (RCU): **0.3** ○ Edge Split **Off** ➢ *Cleanup* : ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**96.0**, ☐max=**96.0** ○ Eccentricity: **not selected** ○ Mean Intensity: **not selected** ○ Integrated Intensity: **not selected**	**Red (Analyze)** ➢ *Parameters:* ○ **Top-Hat** ○ Radius(um): **50** ○ Threshold (RCU): **0.3** ○ Edge Split **Off** ➢ *Cleanup* : ○ Hole fill: **0** ○ Adjust size: **0** ➢ *Filters:* ○ Area (um^2): ☑min=**96.0**, ☐max=**96.0** ○ Eccentricity: **not selected** ○ Mean Intensity: **not selected** ○ Integrated Intensity: **not selected**

Name	Company
pHrodo Red-LDL	ThermoFisher Scientific
HepG2 cells	E. Thomas Lab, U. Miami
MEM	Sigma
Sodium Pyruvate	Sigma
L-Glutamine 200mM solution	Sigma
FBS	Atlas Biologicals
HK2 cells	ATCC
Keratinocyte SFM media kit	Gibco
Primary Coronary Artery Endothelial Cells	ATCC
Vascular Cell Basal Medium	ATCC
Endothelial Cell Growth Kit-VEGF	ATCC
Human Lipoprotein Deficient Serum	Millipore
PBS	Sigma
Trypsin-EDTA (0.25%)	Gibco
Trypsin-EDTA (0.05%)	Gemini Bio-Products
Trypsin Neutralizing Solution	ATCC
24 well plate	Falcon
40 µM mesh cell strainer	VWR
15 mL conical tubes	VWR
50 mL conical tubes	VWR
Trypan Blue Staining (0.4%)	Life Technologies
Counting Slides	Bio-Rad
Incucyte Zoom	Sartorius
Dynasore Hydrate	Sigma
PCSK9 Recombinant Protein	Cayman Chemicals

Catalog Number	Comments
L34356	
HB-8065	
M0325	
P5280	
G7513	
FP-0500-A	
CRL-2190	
17005-042	
PCS-100-020	
PCS-100-030	
PCS-100-041	
LP4	
D8537	
25200056	
400-150	
PCS-999-004	
353226	
10199-654	
89039-666	
89039-658	
T10282	
145-0011	
Zoom	Imaging and analysis platform
D7693	
20631	

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Dear editor,

Please see responses to your comments below.

1. It's fine that you focus on the IncuCyte system here, but do you have specific alternatives you can recommend in the manuscript?

We added to the Discussion the following:

"While this protocol offers specifications for InCucyte live imaging and analysis system, the protocol can be adapted for alternative imaging systems such as Cellomics."

2. Figure 3: There do not appear to be any '####' markings in the Figure itself.

Fixed.

3. The uploaded figure 4 is incorrect.

Fixed.

4. Table 1: Note that Tables will be formatted directly from the excel files for web viewing, and therefore certain formatting choices and special characters may not be displayed correctly. Could this be a Figure instead?

Please continue to use the excel file. We can also send you the table as a pdf but would rather present it as a table instead. Can you please let us know if and which characters would be affected? Please let us know. Thanks.

5. Table 2: There is no 'D' in the table.

Added.