New Method for Real-Time Autophagy Studies

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Autophagy is an intracellular process leading to the lysosomal degradation of cytosolic components and organelles. Its best understood role is in cellular housekeeping; this activity directs the removal of damaged or unwanted products [1]. However, autophagy can also be induced in response to cancer therapies, when autophagy functions as a survival mechanism and thus potentially limits drug efficacy [2,3]. In established tumours, malignant progression and tumour maintenance have been linked to physiological adaptations resulting in upregulated or constitutively active autophagic pathways [2]. In addition, there are many stimuli that have been shown to activate autophagy, including nutrient starvation, reactive oxygen species [4], stress on the endoplasmic reticulum, and ammonia [5].

Once stimulated, unwanted cytosolic proteins and aging organelles are sequestered by a double-membrane vesicle known as an autophagosome *(Figure 1)*. Protein complexes coordinate vesicle formation and enable the recruitment of LC3 into the inner and outer membranes of the autophagosome. LC3-labelled vesicles are trafficked to the lysosome. During this last phase, autophagosomes fuse with lysosomes to form autolysosomes, where unwanted nutrients are reduced to basic molecular building blocks and ultimately released back into the cytoplasm.

Measurement and tracking of autophagy are essential for elucidating this process. Many newer autophagy assays rely on the expression of stably transfected green fluorescence protein (GFP)-LC3 fusion proteins; in this case, autophagosome activity is visually identified by changes in GFP puncta [6]. Lysosomal inhibitors, such as chloroquine (CQ), have also been invaluable in determining the relative autophagic response to cellular stress. CQ blocks the last step of autophagy, lysosomal degradation; the resulting buildup of intermediates can serve as a quantifiable marker of autophagic activity [7]. By combining the use of live cell imaging with transduction of a GFP-tagged autophagosome marker (LC-3) in the presence of CQ, researchers can monitor the autophagosome formation process on a fluorescent microscope in real time. However, little is known about the latter stages of autophagy and the dynamics of lysosomal degradation.

In this article, we demonstrate the use of a microfluidic live cell imaging platform (the CellASIC® ONIX Microfluidic Platform, EMD Millipore) to develop a dynamic cell-based assay for monitoring the whole autophagy process. This platform offers temperature and gas control as well as media perfusion for precise environmental control. Using this system, LC3-GFP CHO reporter cells were subjected to nutrient starvation or hypoxic stresses for a designated time period followed by reintroduction of normal growth conditions. The time course of autophagy was visualised in real time under a fluorescent microscope, providing quantitative information on both autophagosome formation and lysosomal degradation machinery.

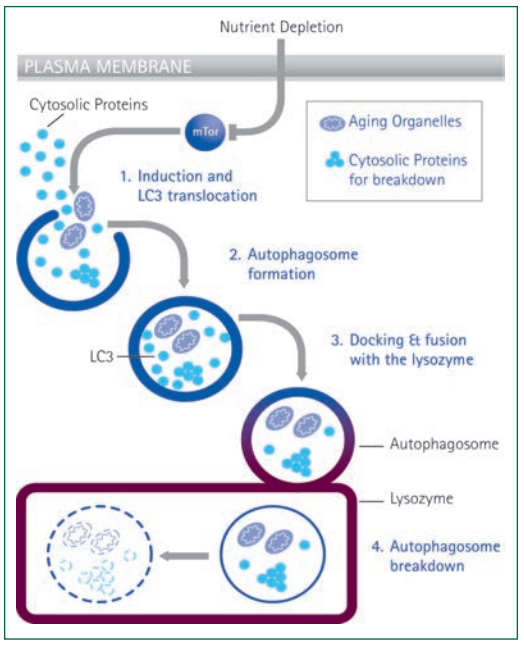

Figure 1. Four stages of autophagy. Autophagy can be induced by nutrient depletion or inhibition of the mTOR pathway. During autophagy, cytosolic proteins and aging organelles are sequestered by a double-membraned autophagosome. One of the hallmarks of autophagy is translocation of LC3 from the cytoplasm to the autophagosome. Autophagosomes then fuse with lysosomes to promote breakdown of the vesicle and all contents, including LC3. This process can be visualised using either a LC3-GFP fusion protein or an anti-LC3 antibody.

Assay Validation

To validate the media exchange capability of the CellASIC® ONIX platform as well as the ability to monitor and quantify autophagy through autophagosome counting, LC3-GFP CHO cells (70% confluent) were perfused with EBSS + 50 µM CQ for 100 minutes followed by regular culture medium for 200 minutes. As shown in *Figures 2 and 3*, the dynamic changes of autophagy in both the stress and recovery phase could be quantified through autophagosome counting.

Assessment of CQ Dose Response

Once the assay was validated, profiling of the CQ dose response in CHO cell lines was conducted. Once established in the microchamber, exposure conditions involved three phases: standard culture medium for 135 minutes, continuous CQ (10 µM, 100 µM, or 1 mM) perfusion for 255 minutes, and culture medium for the final 240 minutes to permit visual capture of the lysosomal degradation process. Images were taken every 15 minutes. Overall, the rate of autophagosome formation was proportional to the CQ concentration applied. However, at 1 mM, cells ceased committing to the autophagy pathway, and the number of autophagosomes stayed constant for the rest of the experiment. We also observed more dead cells in this treated group, indicating either that the maximal levels of autophagy in this cell line had been achieved, or that the cells committed to apoptosis or necrosis at the high CQ dose. Furthermore, degradation of autophagosomes occurred at a faster rate than the accumulation *(Figure 4)*.

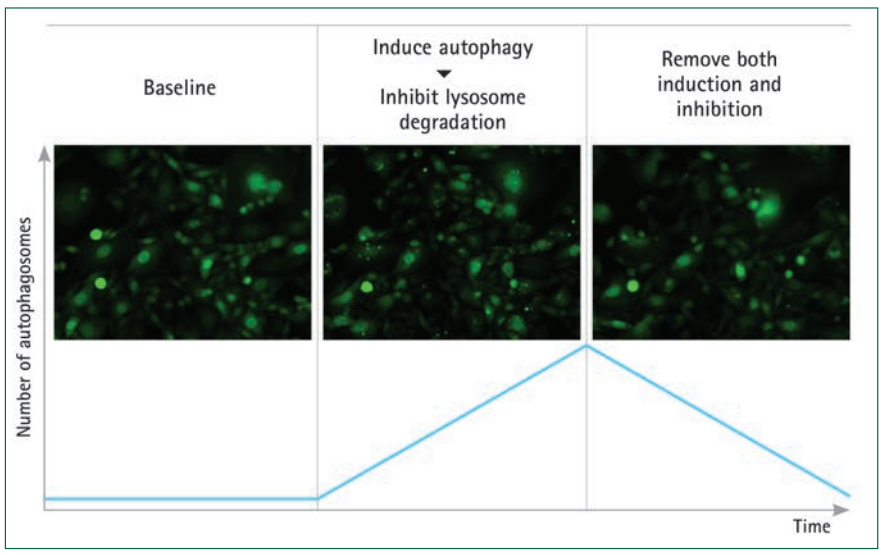

Figure 2. Schematic of live cell imaging for autophagy of LC3-GFP expressing CHO cells. First, medium was perfused to establish fluorescent baseline. A stressor (serum starvation) and the lysosome inhibitor CQ1 were then introduced to trigger autophagosome accumulation within cells. When cells were returned to standard growth medium, autophagosomes underwent lysosomal degradation.

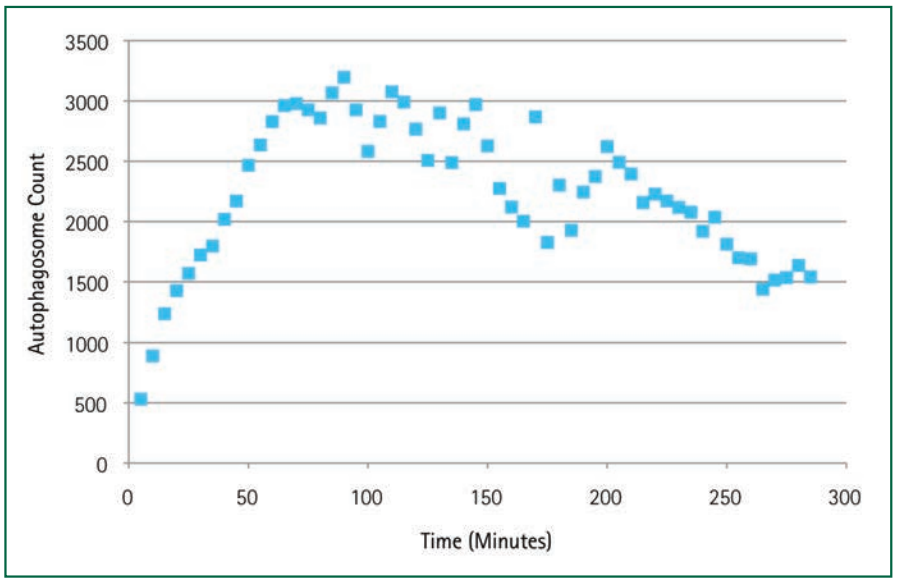

Figure 3. Validation of the autophagosome counting assay. The number of autophagosomes in each image in Figure 2 was determined using a custom-developed image processing sequence for object identification with CellProfiler Software. Rate of autophagosome formation and degradation was successfully monitored with the proposed assay.

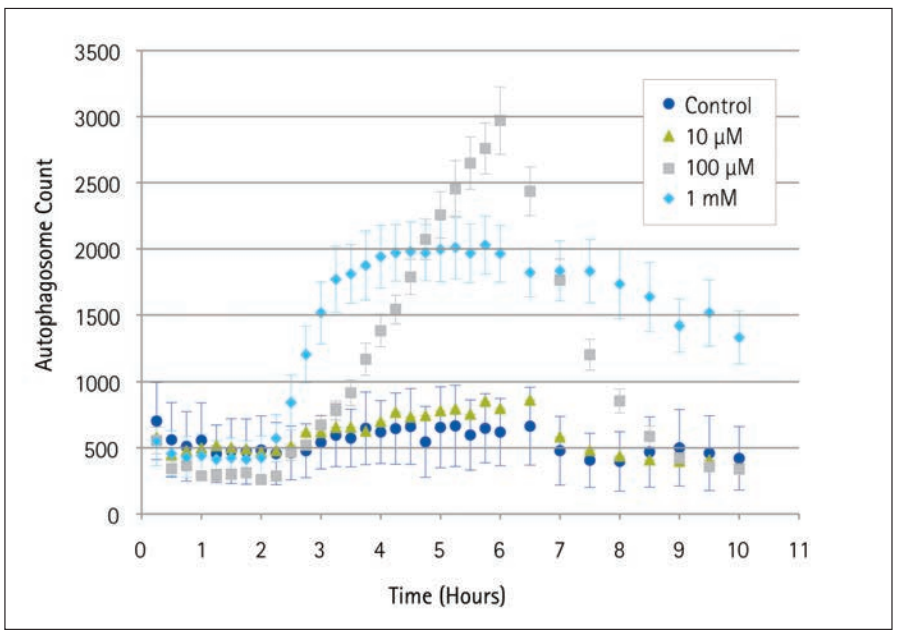

Figure 4. Assessment of CQ dose response using a dynamic autophagy assay. Three levels of CQ (10 µM, 100 µM, and 1 mM) were perfused through independent culture chambers of the same microfluidic plate at the same time. Time-lapse imaging was performed on three different positions in each of the microchambers. The fluorescence intensity was counted and averaged per frame using CellProfiler Software and normalised to the background to measure flux. Error bars represent standard deviation (S.D.) of the number of counted puncta in approximately 60 cells per time point.

Hypoxia Studies

To further explore the dynamics of stress-induced autophagy, we exploited the CellASIC® ONIX system's ability to regulate gaseous microenvironments to introduce severely hypoxic conditions within the cell chamber. Prior to analysis, the system's control of oxygen content was validated. For gas flow rates of 20 mL/min and 3 mL/min, we consistently found that the switch time from normoxic to hypoxic gas environment occurred in less than one hour. For these two gas flow rates, steady-state concentrations were achieved with less than 2% and 10% deviation from the supplied gas, respectively.

In traditional static cultures, achieving equilibrium following defined gas switching is impractical due to incubator size and differences between the measured pericellular oxygen tension (within the flask) and that in the ambient air [8,9]. However, the new platform features a significantly reduced culture vessel size (10,000 cells per chamber) and restricted fluid volume (a few nanolitres), together leading to a faster gas exchange during our hypoxia studies.

Results from initial hypoxia experiments supported this fact; specifically, we found that, compared to the typical hypoxic response of cells cultured in traditional petri dishes [8-12], LC3-GFP CHO reporter cells in the microfluidic perfusion environment were far more sensitive to gas switching, demonstrating autophagosome formation within three hours of hypoxic treatment [10-12]. Following six-hour exposure, a large percentage of cells failed to recover and underwent apoptosis.

Profiling Autophagosome Formation

Based on these preliminary results, we performed dynamic profiling of autophagosome formation in reporter cells in response to CQ (10 µM, 100 µM, or 1 mM) under hypoxia conditions. Similar to results of starvation-induced autophagy, the rate of autophagosome appearance accelerated with respect to increasing CQ dose. As for the recovery phase, cells treated with 100 µM of the CQ responded almost instantaneously, while those treated with the highest dose (1 mM) demonstrated a far more protracted recovery profile *(Figure 5 and 6)*.

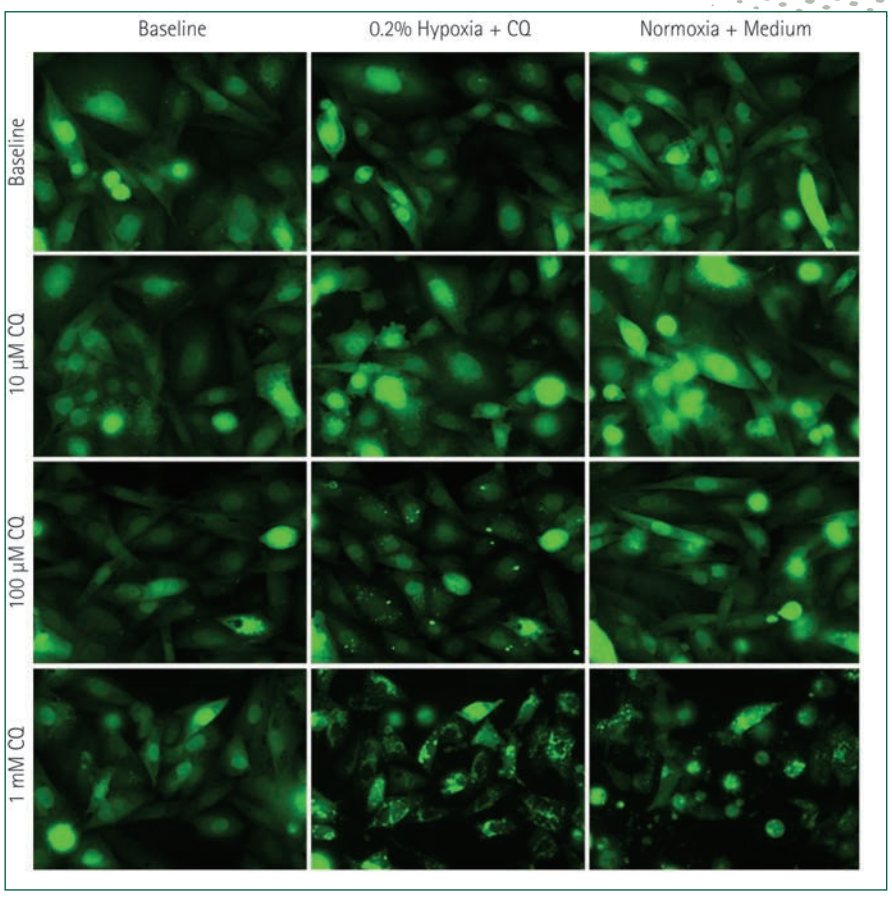

Figure 5. Images of the LC3-GFP CHO reporter cells taken during each phase of the hypoxia-induced autophagy assay. Cultures were first perfused with standard growth medium under normoxic conditions for 60 minutes, followed by continuous CQ (10 µM, 100 µM, or 1 mM) perfusion in the presence of severe hypoxia (0.2% O_2) treatment for three hours, followed by removal of the stressors and reestablishment of normoxia in standard growth medium for another 16 hours. An Olympus IX-71 inverted microscope was used for the entire process; all images were taken under the 40x objective.

To simultaneously monitor the two most important organelles involved in autophagy, we further transduced the LC3-GFP reporter CHO cells with a fluorescently tagged, lysosome-specific fusion protein construct, LAMP1-RFP. Transduced cells were incubated under mildly hypoxic conditions (3% O_2) in the presence of CQ at various concentrations for 180 minutes, followed by prolonged 660-minute culture under normoxia in the presence of standard medium.

The data indicate that autophagogome formation started immediately after the switch to hypoxic conditions and lasted for three hours in the cells treated with 1 mM of CQ. In these cultures, lysosome degradation did not occur until almost 11 hours after gas exchange *(Figure 7)*. However, we did not observe any conclusive response in the lysosomal activity during either autophagy or recovery phases except for the observation that lysosomes were instantly condensed under the hypoxic stress.

We speculate that the LAMP1-RFP transduction process (or the LAMP1-RFP construct itself) might be another source of cellular stress, hence affecting overall autophagic activity. We are currently exploring alternative labelling methods for dual-colour assays for hypoxia-induced autophagy.

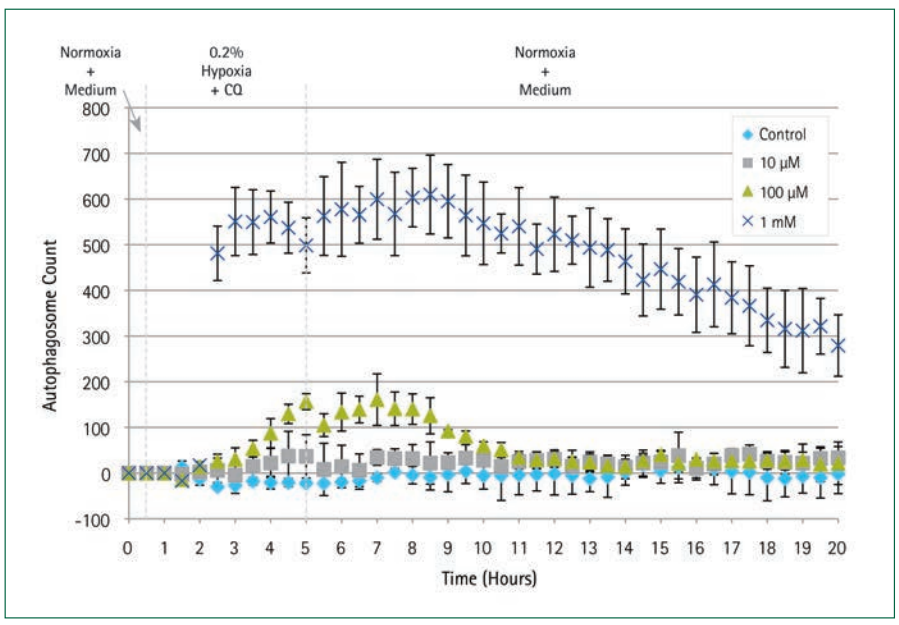

Figure 6. Dynamic, quantitative live cell imaging of autophagosome formation with respect to CQ treatment and hypoxia. Three levels of CQ inhibitor (10 µM, 100 µM, and 1 mM) were perfused through independent culture units at the same time. Time-lapse imaging was performed on three different positions in each of the microchambers. The fluorescence intensity was counted and averaged per frame using CellProfiler Software and normalised to the background to measure flux. Error bars represent S.D. of the puncta in around a total of 60 cells per time point.

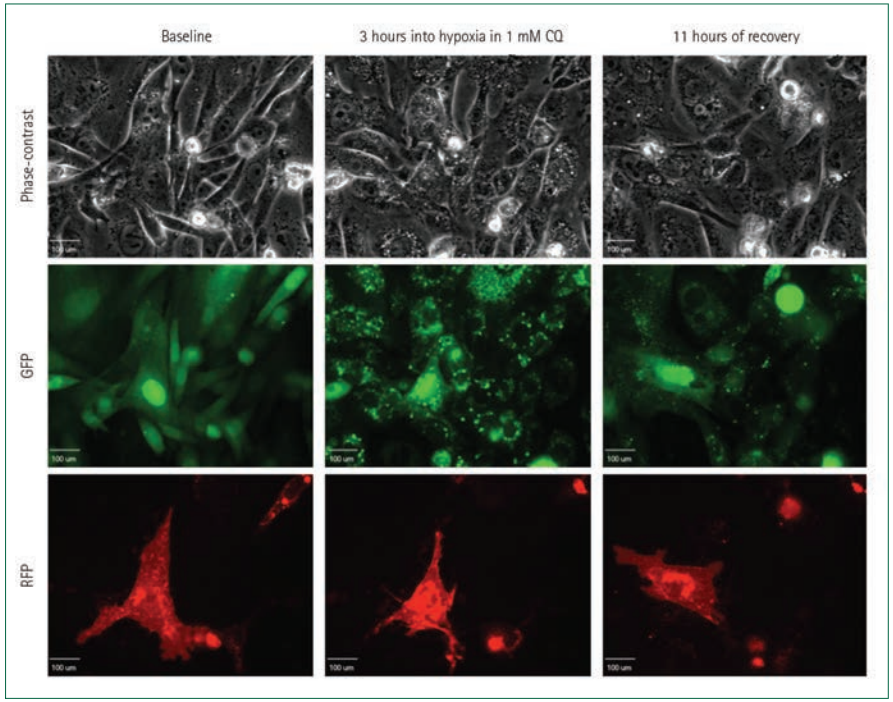

Figure 7. Two-colour imaging of transduced LAMP1-RFP/LC3-GFP CHO reporter cells showing autophagosomes (green) and lysosomes (red) throughout the entire hypoxia-induced autophagy assay. Cells were first treated with mild hypoxia (3%) for three hours in the presence or absence of CQ, followed by the removal of the stressors and the treatment of normoxia and the growth medium for another 16 hours. An Olympus IX-71 inverted microscope was used for the entire process, and all images were taken under the 40x objective.

Conclusion

The CellASIC® ONIX live cell imaging platform was used to create a dynamic assay that not only has the potential to simultaneously monitor multiple intracellular components throughout the entire autophagic process without disruption but also allows precise manipulation of culture parameters, thus exposing cells to more physiologically relevant conditions. This platform may be capable of simulating conditions of pulse exposure to drug compounds, and could provide additional information on dose response for compound profiling by revealing rates of autophagosome formation and degradation. It therefore has the potential to help in the discovery of new targets and therapeutic compounds in cancer as well as other diseases.

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