Development of a Cellular Platform to Study Lysosomal Functions in Neurodegeneration

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Introduction

Several neurodegenerative disorders converge on the lysosome, with genetic links to endo-lysosomal dysfunction in both familial and sporadic forms of disease (for example LRRK2 mutations in Parkinson's disease (PD), GBA mutations in PD, Gaucher's disease, and dementia with Lewy bodies, GRN in frontotemporal dementia, and PLD3, BIN1, RIN3, APOE in Alzheimer's disease). In vitro and in vivo disease models, and post-mortem tissue also show strong evidence of lysosomal dysfunction. As well as playing a role in intracellular signalling and homeostasis, lysosomes are a key site of protein degradation, both of intracellular material via autophagy, and of extracellular endocytosed material. Altogether this makes lysosomes an attractive therapeutic target for neurodegenerative diseases. We have established cell-based assays of lysosomal functions and autophagy for use in drug discovery. The assays are established in multi-well format enabling simultaneous multiple compounds testing. High content confocal imaging assays are performed on the Opera Phenix HCI (PerkinElmer).

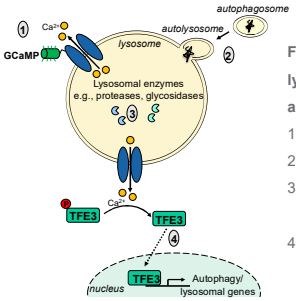

Figure 1. Schematic illustrating lysosomal functions measured by our assay platform:

1. Lysosomal calcium flux
2. Autophagic flux
3. Lysosomal activity (DQ-BSA and GCase)
4. TFE3 nuclear translocation

1. Lysosomal calcium flux

Lysosomal Ca^{2+} signalling plays an important role in a variety of cellular processes including autophagy, endocytosis, and the regulated release of exocytotic vesicles. Cell models using genetically encoded calcium indicators (GECIs)[1] fused to lysosomal calcium selective ion channels facilitate investigations into the role that lysosomal Ca^{2+} homeostasis plays in mediating these cellular processes.

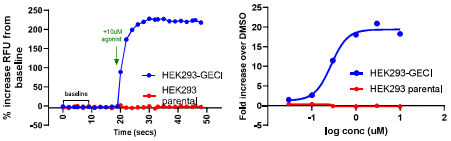

Figure 2: Genetically encoded calcium indicators report calcium flux at the lysosome. HEK293 cells stably expressing GECIs fused to a lysosomal directed Ca^{2+} selective ion channel were compared with parental HEK293 cells via fluorescence imaging (FlexStation) in response to dose-dependent channel activation.

2. Autophagic flux assay

To measure autophagy flux, a stable HeLa cell line expressing tandem RFP-GFP-LC3 was used. The RFP-GFP-LC3 assay exploits the properties of GFP and RFP; GFP fluorescence is quenched by the low pH of the lysosome when autophagosomes fuse with lysosomes, while RFP fluorescence is more stable in acidic compartments, which means that autophagosomes are labelled yellow (green and red merge) and autolysosomes are red only.

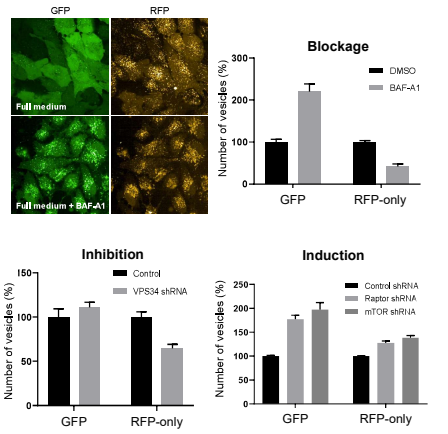

Figure 3. Distinguishing between autophagy modulators with different modes of action.
Quantification of GFP- and RFP-positive puncta enables the identification of modulators that block, inhibit or induce autophagy in a robust manner. Bafilomycin A1 (BAF-A1), VPS34 shRNA, Raptor and mTOR shRNA are shown as positive controls.

3. Lysosomal activity assays

DQ-BSA activity assay

Lysosomal protease activity can be measured using the fluorescent substrate DQ-BSA[2]. The self-quenched substrate is endocytosed and trafficked to lysosomes. Lysosomal proteases degrade the BSA removing the quencher resulting in increased fluorescence.

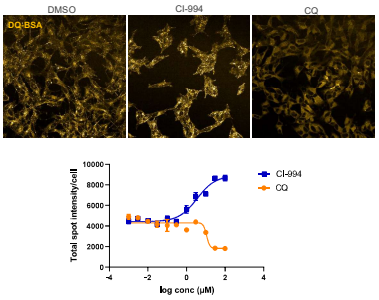

Figure 4: DQ-BSA fluorescence intensity reports protease activity. SH-SY5Y cells were loaded with DQ-BSA. Fluorescence is reduced by 24 h treatment with the lysosome inhibitor chloroquine (CQ), or increased by the histone deacetylase inhibitor CI-994.

GCase activity assay

Glucosylceramide is cleaved in the lysosome to ceramide and glucose by the lipid hydrolase acid-β-glucosidase 1 (GCase). To measure GCase activity, cells are loaded with a quenched fluorescent substrate probe, which localises to lysosomes. Active GCase hydrolyses the probe removing the quencher, resulting in fluorescence increase[3].

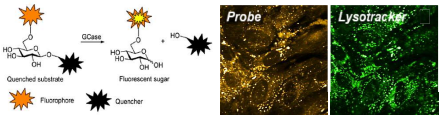

Figure 5: The fluorescent probe is located to lysosomes. Fluorescence probe (yellow) co-localises with lysosomes stained with lysotracker (green).

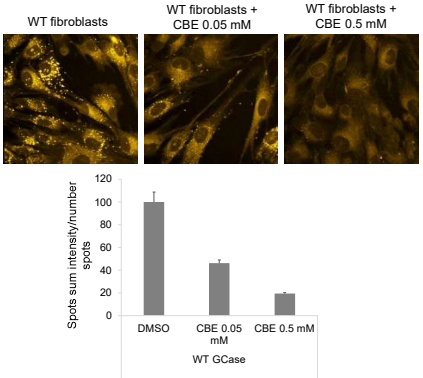

Figure 6: Fluorescence intensity reports GCase activity. Fluorescence is reduced by the GCase inhibitor conduritol-β-epoxide (CBE).

4. TFE3 nuclear translocation assay

Transcription factors TFEB/TFE3 are master regulators of lysosomal biogenesis and drive coordinated expression of autophagy and lysosomal genes. Nuclear translocation of TFEB/TFE3 is a potential readout of increased lysosomal gene expression.

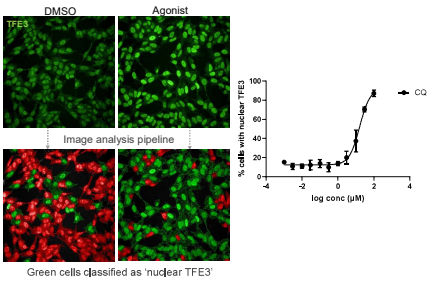

Figure 7: Induction of TFE3 nuclear translocation. SH-SY5Y cells treated for 2 h with CQ, followed by immunostaining with anti-TFE3 antibody. Automated image analysis quantifies the number of cells displaying translocation of TFE3 to the nucleus.

CNS-relevant cell cultures

Cultures of patient and control derived iPSC neurons and glial cells have been established and validated to enable investigations of lysosomal functions in CNS-relevant cell types.

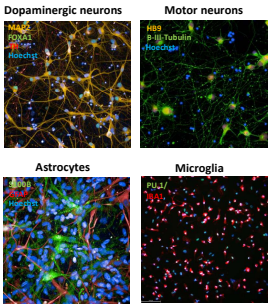

Figure 8: iPSC derived iCells (FujiFilm) were cultured according to the relevant protocols, fixed and stained for cell type markers to confirm their identity.

Summary

We have developed a high-throughput platform that enables comprehensive profiling of lysosomal functions in a range of cell types. The assays can be used to assess the ability of compounds to modulate lysosomal targets and interrogate mechanism of action. Next, we plan to extend the platform to measure additional functions such as lysosomal pH and exocytosis, and assess lead compounds in iPSC-derived cells. Identifying compounds that improve lysosomal functions may lead to the development of novel therapeutics for neurodegenerative diseases.

References:
1. Nakai et al., Nature Biotechnology, 2001, 19, 2, 137–41
2. Carling et al., Autophagy, 2023, 19, 2, 692-705
3. Yadav et al., J. Am. Chem. Soc, 2015, 137, 3, 1181–1189

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