APPLICATION NOTE

HTRF analysis and imaging of Phospho-S65-Ubiquitin in cellular models of mitophagy.

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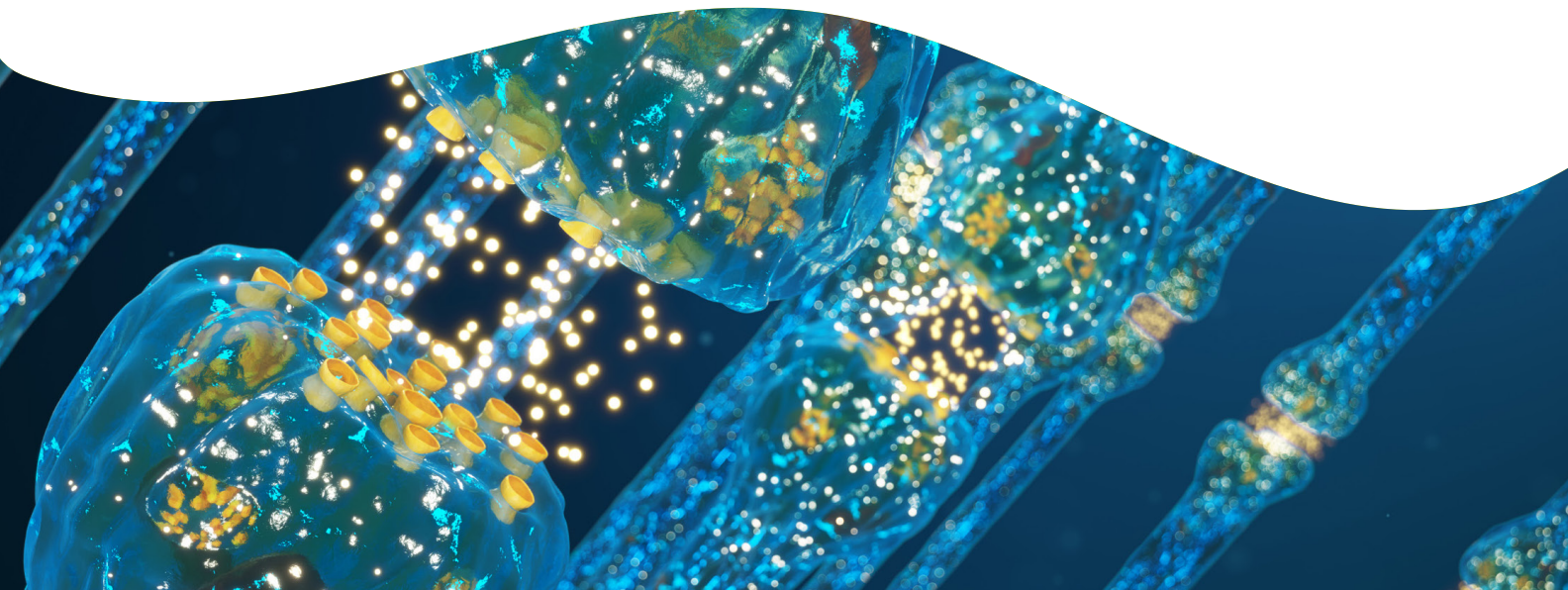
Introduction

The scientific community has demonstrated over the past years the multifactorial etiology of the most common neurodegenerative diseases, and although intense efforts have enabled the ability to shed light on important molecular mechanisms, the entire pathogenesis of these disorders remain to be elucidated. Recent research studies have established that defective mitophagy constitutes one of the hallmarks of several neurodegenerative disease onsets, opening new opportunities for therapeutic intervention. Mitophagy is a sophisticated regulatory pathway developed by cells to maintain mitochondrial homeostasis and quality control, enabling the degradation of damaged mitochondria. This evolutionarily conserved cellular process is significantly impaired in neurodegenerative disorders such as Parkinson's and Alzheimer's disease, leading to neuronal death (Figure 1).

Ubiquitin phosphorylation at Serine 65 has been shown to be a specific marker of Ubiquit-independent PINK1/Parkin-mediated mitophagy induction (Figure 2).

Revvity has developed the first HTRF® (homogeneous time-resolved fluorescence) no wash Phospho Ubiquitin Serine 65 immunoassay specifically for studying mitophagy activation.



Mitophagy in neurosciences

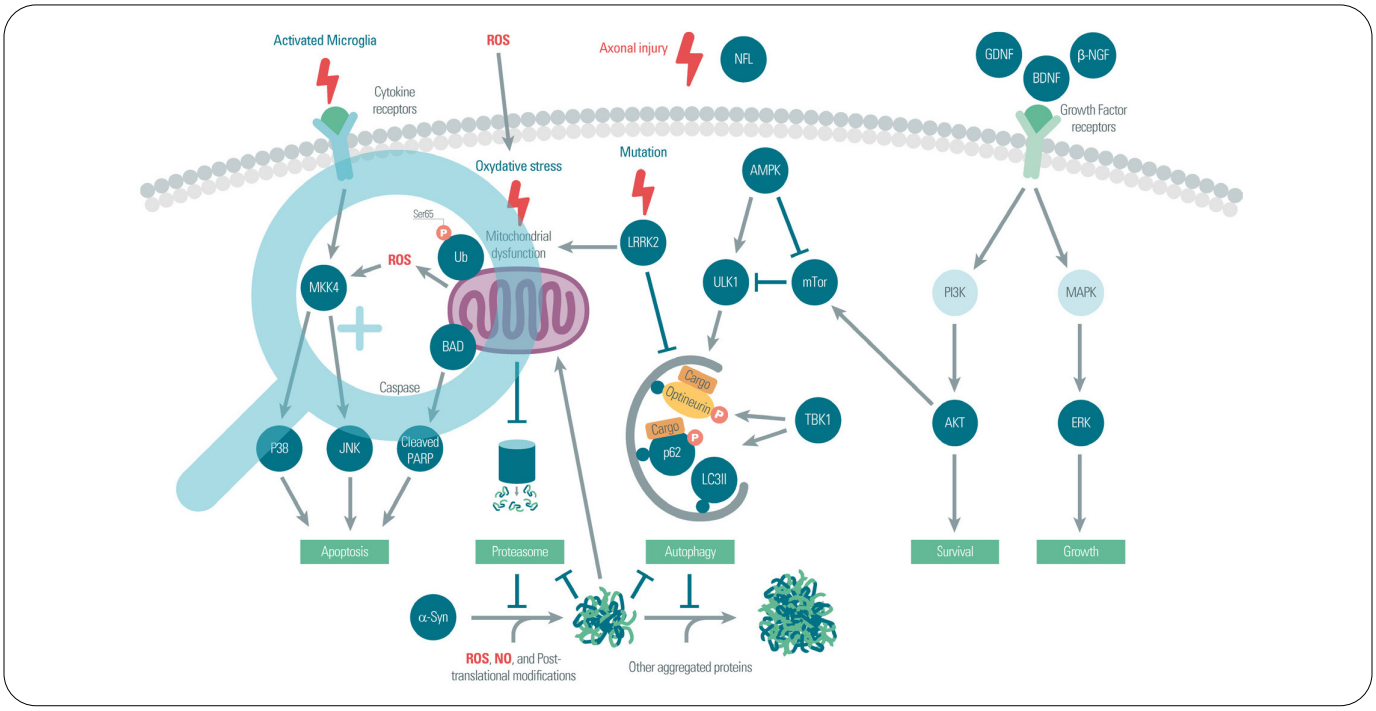


Figure 1. Most prominent molecular pathways in early-onset familial Parkinson's disease.

Ubiquitin and PINK1/parkin pathway in mitophagy

PINK1 phosphorylates Parkin on Serine 65 in the N-terminalUbiquitin-like domain[1] as well as other ubiquitinated species.Phospho-S65-Ubiquitin (pSer65-Ub) is barely detectable (mass spectrometry) in cells lacking PINK1 and is < 0.1% of total ubiquitin in wild-type cells without mitochondrial depolarization.It can rise to more than 2% of the total ubiquitin pool in cells after CCCP-induced depolarization.[2] PINK1, pSer65-Ub, and Parkin form a positive feedforward amplification loop to initiate mitophagy. The PINK1/Parkin signaling is linked to Parkinson's disease[1,2] and either PINK1 or Parkin are mutated[1] in many cases of early-onset familial Parkinson's disease.

Upon mitochondrial damage, such as loss of membranepotential (induced by mitochondrial electron transportdecoupling agents such as CCCP or valinomycin), PARL-dependent cleavage of PINK1 is inhibited and PINK1 accumulatesat the outer membrane surface of damaged mitochondria flagging it for removal. PINK1 has been shown to phosphorylate ubiquitin[3] on the outer mitochondrial membrane (Figure 2), which then binds to and activates the ubiquitin ligase Parkin, causing cytosolic Parkin to translocate to the surface of the mitochondria.[4] Parkin then increases the level of ubiquitination of outer mitochondrial proteins, creating a positive feedback loop. The ubiquitin chains thus generated can interact with autophagy adaptors leading to mitophagy.

Ubiquitin itself is a small (8.6 kDa) regulatory protein which is present under the form of many identities within a cell. Most proteins will experience ubiquitination at some point in their cellular lifetime, resulting in degradation by the proteasome. Ubiquitin is attached to substrates by a sophisticated enzymatic cascade involving E3 ubiquitin ligating enzymes.

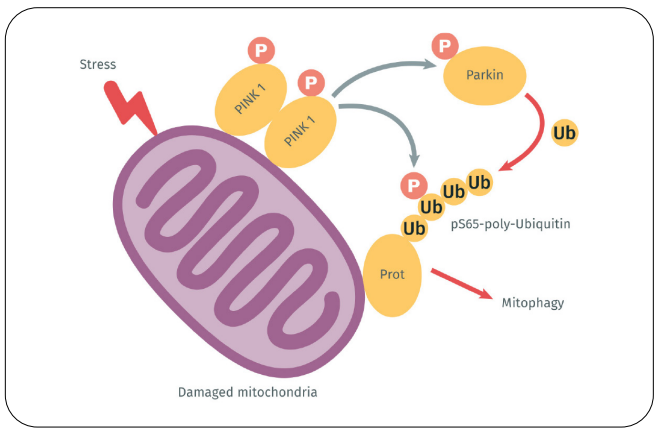


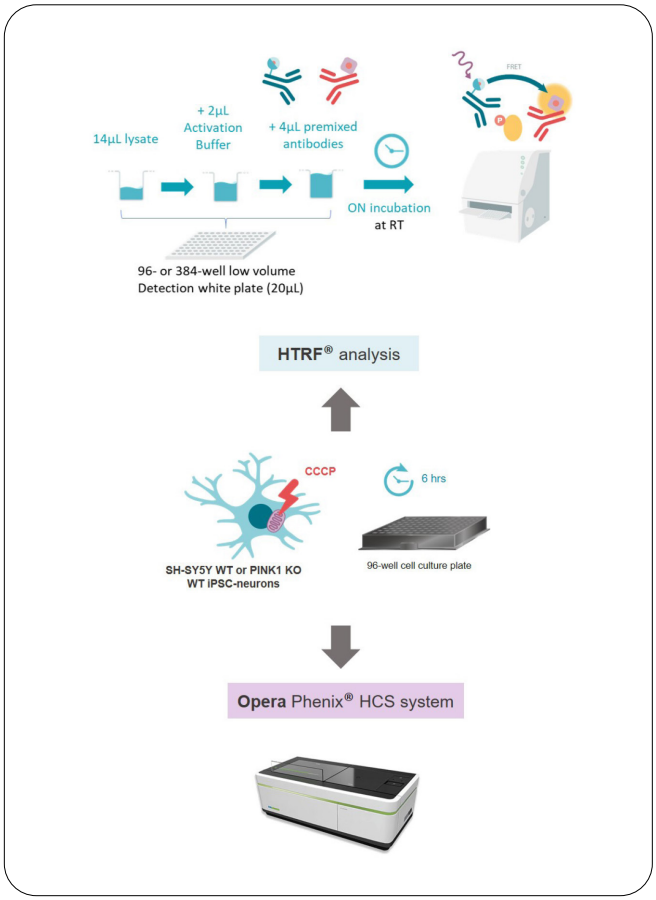
Figure 2. Focus on mitophagy and PINK1/Parkin signaling pathway.

Ubiquitinated proteins are recognized by receptors that contain ubiquitin-binding domains and several specialized families of proteases, known as the deubiquitinases (DUBs), remove ubiquitin modifications (for a review see Reference 4).

The 'unanchored' ubiquitin or ubiquitin chains perform secondmessenger-like functions, mono-ubiquitin is a prominent component of cell lysates.[5] Phospho-S65Ub can be found as Lys48- and Lys63- linked chains, the reference method for identification and quantification being mass spectrometry-based methods.[6,7]

Materials and methods

Experimental flowchart



Cell Models, reagents and consumables

- Human neuroblastoma SH-SY5Y Wild Type (ATCC® #CRL-2266)
- SH-SY5Y PINK1 Knock-out (Department of Physiology, Anatomy and Genetics / University of Oxford, UK)
- Wild Type iPSC-neurons Department of Physiology, Anatomy and Genetics / University of Oxford, UK)
- DMEM/F-12 (1:1) HEPES (GIBCO# 31330-038) + 10% FBS + 1% P/S.
- Fetal bovine serum (FBS), heat inactivated (Thermo Fisher, #10082-147)
- Proxiplate-384 plus (Revvity, #6008280)
- HTRF Phospho-Ubiquitin (Ser65) kit (Revvity, #64UBIS65PEG)
- HTRF Alpha-Tubulin Housekeeping kit (Revvity, #64ATUBPEG)
- Carbonyl Cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, #C2759) 100 mM stock solution in DMSO
- Valinomycin (Sigma-Aldrich, #V0627) 20 mM stock solution in DMSO

Assay technologies

HTRF is a TR-FRET based technology and stands for homogeneous time resolved fluorescence. It is based on the fluorescent resonance energy transfer (FRET) between two fluorophores, a donor and an acceptor. These fluorophores can be coupled to antibodies targeting an analyte such that once bound they come in proximity to one another. Excitation of the donor by an energy source (e.g.a flash lamp or a laser) triggers an energy transfer towards the nearby acceptor, which in turn emits specific fluorescence at a given wavelength (Figure 3).

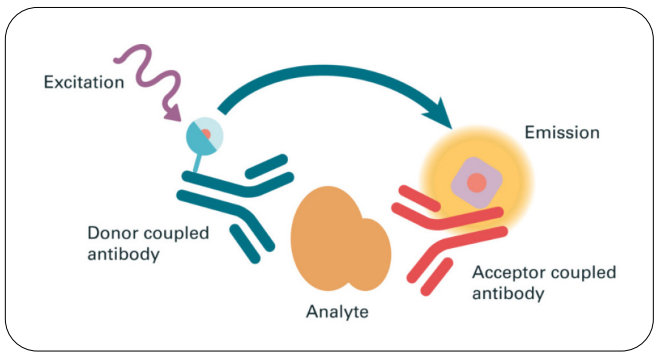


Figure 3. HTRF Assay Principle. When the labelled antibodies bind to the same antigen, the excitation of the donor with a light source (laser or flash lamp) triggers a fluorescence resonance energy transfer (FRET) to the nearby acceptor, which in turn fluoresces at a specific wavelength. The two antibodies bind to the analyte present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the analyte concentration.

Cell culture, treatment, and sample generation

SY-SY5Y neuroblastoma wild-type or PINK1-KO cells were seeded at 105 cells/well in a 96-well plate and incubated overnight in DMEM:F12 + 10% (v/v) FBS + 5 mM L-glutamine + 1% (v/v) penicillin/streptomycin for cell attachment. The cells were either kept untreated, treated with DMSO alone, or submitted to CCCP concentrations ranging from 2.5 to 30 µM final in wells for six hours at 37 °C with four wells per condition (final DMSO concentration of 0.03%). Wells were washed two times with cold PBS and lysed with 50 µL lysis buffer #1 supplemented with blocking reagent for one hour at room temperature.

For imaging, SH-SY5Y cells were seeded in a CellCarrier-96 Ultra plate and after overnight adhesion, the cells were treated with either DMSO or increasing CCCP concentrations ranging from 5 to 30 µM. After 4% PFA/PBS fixation (10 minutes at room temperature), permeabilization (Triton-X100), and saturation steps (5% BSA for one hour), the cells were incubated with anti-pS65Ubiquitin Merck/Millipore ABS1513-I (1/1000) overnight at 4 °C followed by incubation with a secondary Alexa Fluor 488 antibody and DAPI (1:1000) for one hour. Cells were imaged using an Opera Phenix High Content Imaging System (Revvity).

iPSCs reprogrammed from a healthy donor were differentiated towards a dopaminergic neuronal culture, as previously described.[8] iPSC-neurons were seeded 3 x 10^4 cells/well in a 96-well half-area plate, incubated overnight, and then treated in supplemented neurobasal medium for 6 hours with 5-10 µM CCCP with a final DMSO concentration of 0.03% (v/v).

Ten replicate wells were assayed per condition. Wells were washed twice with cold PBS and lysed with 25 µL of supplemented lysis buffer #1 for one hour at room temperature.

SH-SY5Y cells were seeded (2 x 10^5 cells/well) in a 96 -well plate and cultured overnight in complete culture medium as above.Medium was renewed and the cells were incubated for four hours with increasing concentrations of valinomycin. After medium removal, the cells were then lysed with 50 µL of 1X supplemented lysis buffer #1 for 30 minutes at room temperature.

HTRF Data collection and analysis

HTRF assays were performed following each kit's recommended protocol. Briefly, lysate samples (14 µL, neat or prediluted in lysis buffer) were transferred in triplicate to a white 384 well low volume plate together with negative controls containing 14 µL of lysis buffer. Two microliters of Activation Reagent were distributed to each well, followed by 4 µL of premixed pS65Ubiquitin detection antibodies (#64UBIS65PEG). For convenience, 6 µL of extemporaneous premix of Activation Buffer and detection antibodies can be distributed.

In parallel, 4 µL of lysates were transferred to a similar plate followed by 12 µL of supplemented diluent #8 and 4 µL of premixed alpha-tubulin detection antibodies (# 64ATUBPEG) according to the kit protocol.

The plates were incubated overnight at room temperature. The pS65Ubiquitin and alpha-tubulin HTRF signals were measured on an HTRF compatible reader. Upon excitation, emissions at 620 nm (europium donor reference signal) and 665 nm (d2 acceptor signal) were recorded. Data is reported as the HTRF ratio of acceptor to donor signal = (665/620)*10,000. The measurement of intracellular phospho-S65-Ubiquitin is a relative quantification where the HTRF signal intensity (HTRF Ratio) is directly proportional to the concentration of the phosphorylated or total protein in the cell lysate.

Data were analyzed in GraphPad Prism software. Semi-quantitative data in figures represent the mean +/- SD (Standard Deviation) of each experimental replicate.

Results

Image analysis from Opera Phenix data on SH-SY5Y cells revealed characteristics punctuated by increased staining of phosphorylated ubiquitin (in green) correlated with the CCCP concentration (Figure 4). The vehicle, DMSO condition, only reported signals in the DAPI channel. The observed ubiquitin phosphorylation is related to an expected CCCP-induced mitophagy that is well established and described for this reference compound.[9] This imaging data clearly validates that the cell model and CCCP compound treatment set-up were effective in inducing mitophagy.

The lysates generated from CCCP-stimulated 100K SH-SY5Y WT cells per well (96-well plate) lysed in 60 µL of supplemented LB#1 were enough to generate a robust pS65-Ubiquitin HTRF signal with a signal over noise ratio of 10.

The tubulin signals were constant, showing that there were no issues with CCCP treatment up to 15 µM such as cell detachment or toxicity (Figure 5). The pS65Ubiquitin signal was plotted against CCCP molar concentration (log scale). The error bars on the graph correspond to an average of twelve biological replicates. For the wildtype SH-SY5Y cells, phospho-S65Ubiquitin concentration increased according to CCCP concentration with an EC_{50} around 3 µM in accordance with reported values for CCCP induced mitophagy (see references and notes). Moreover, the HTRF data reported here was completely in-line with the effects observed on cells with imaging, generating a convincing dataset for assay validation with this kit being the first commercially available immunoassay to monitor ubiquitin phosphorylation, apart from complex and expensive mass spectrometry or poor accuracy western blotting techniques.

In order to demonstrate specificity of the HTRF phospho-ubiquitin kit, the same experiment was carried out on a PINK1 KO from the same cell line. As PINK1 is the upstream kinase implicated in the ubiquitin phosphorylation cascade, the impairment of this signaling is known to suppress ubiquitin phosphorylation. As expected for PINK1-KO cells, the pS65Ubiquitin signal was at the baseline level showing that the PINK1/Parkin signaling is inhibited and in turn the ubiquitin is no longer phosphorylated. Aside from validating the excellent specificity, this result also demonstrated the usefulness of this kit to monitor compounds that are known to modulate the PINK1/Parkin pathway.

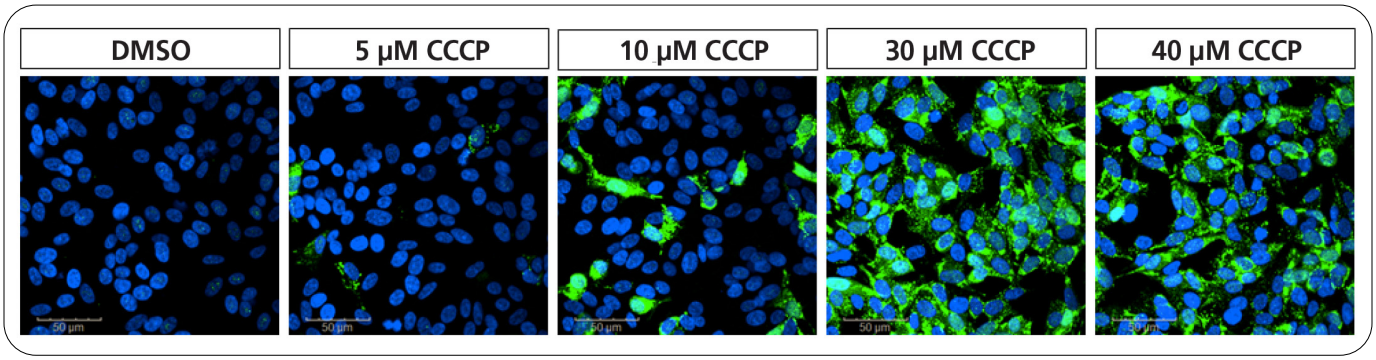


Figure 4. IF analysis of pS65Ubiquitin increase on SH-SY5Y cells (nuclei and phospho-ubiquitin are reported here in blue and green respectively). Acknowledgments to Brent Ryan & Ana Belen Malpartida / OPDC / University of Oxford UK.

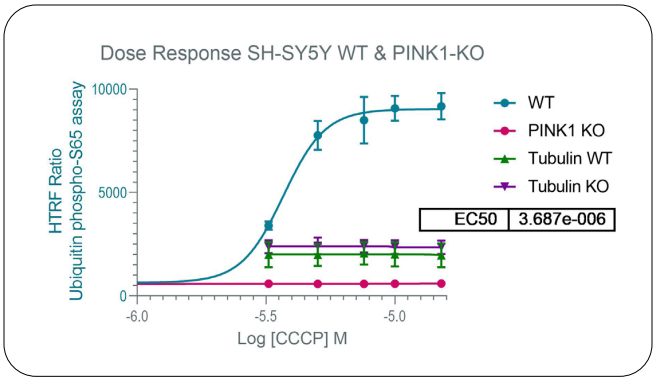


Figure 5. HTRF analysis of pS65-Ubiquitin on WT and PINK1-KO SH-SY5Y cells. Acknowledgments to Brent Ryan & Pavandeep Rai / OPDC /University of Oxford.

Valinomycin, a selective K^+ ionophore known to induce mitochondrial membrane depolarization, promoted ubiquitin phosphorylation on Ser65, with an EC_{50} in the nanomolar range (Figure 6). The reported valinomycin EC_{50} is 5.5 nM for mitochondria membrane depolarization. This observation agrees with the valinomycin induced mitophagy at submicromolar concentrations,[10] further validating the accuracy of the new pSer65-Ubiquitin HTRF kit to perform pharmacology experiments on cellular mitophagic models.

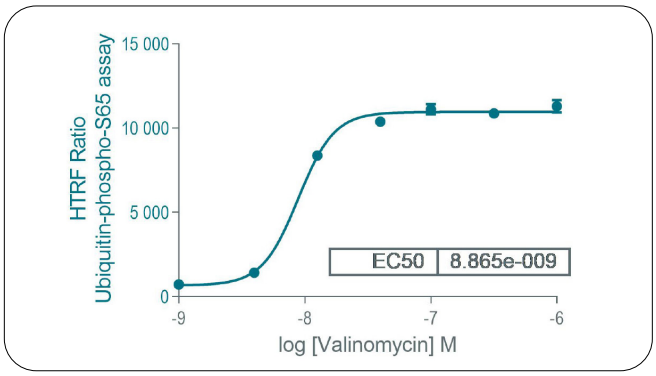


Figure 6. HTRF analysis of pS65-Ubiquitin of valinomycin induced mitophagy on SH-SY5Y cells.

After having validated the CCCP-induced mitophagy in SH-SY5Y WT and PINK1 KO cells with imaging and HTRF, the phospho-ubiquitin HTRF kit was challenged in more phenotypic and relevant cell models such as iPSC derived neurons. In this cellular model, the tubulin signal was also constant, showing that there were no CCCP toxicity issues concerning iPSC. The pS65Ubiquitin signal was plotted against CCCP concentration (Figure 7). The EC_{50} was roughly in the 5 µM concentration range as expected and in accordance with literature reported values. Even with a low concentration of cells per well (30,000), we were able to detect ubiquitin phosphorylation with a signal to noise ratio of 10.8 at a concentration of 5 µM close to the EC_{50}.

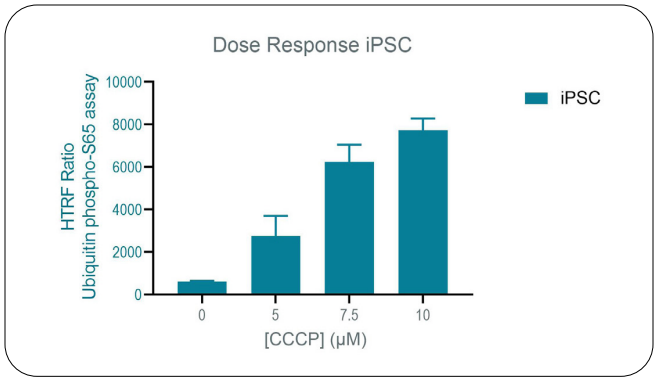


Figure 7. HTRF analysis of pS65-Ubiquitin of CCCP induced mitophagy on iPSCderived macrophages. Acknowledgments to Brent Ryan & Maria Claudia Caiazza / OPDC /University of Oxford.

This result shed light on the possibility of using the HTRF ubiquitin phosphorylation kit with relatively low cell concentrations of highly relevant cell models. With iPSC cells being costly to generate HTS campaigns, it is of importance to have detection tools with sensitivities that are in accordance with the use of low cell concentrations.

Summary

In the current application note, we demonstrate that the HTRF Phospho-Ubiquitin (Ser65) Cellular Kit allows the detection of the CCCP-induced mitophagy signaled by an increase of ubiquitin phosphorylation on native cell lysates. This assay correlates well with the phospho-S65Ubiquitin imaging data generated on the Opera Phenix Imaging System on the same cell model. The CCCP EC50 values in the micromolar range correlate with the values reported in literature using complex techniques.

Validation was done on cellular models that are commonly used in mitophagy, neuro-inflammation, and neuro-degenerative diseases, underlying the usefulness of this new detection reagent in these important therapeutic areas.

The new Revvity | Revvity HTRF Phospho-Ubiquitin (Ser65) Cellular Kit takes analysis efficiency, specificity, and accuracy to the next level compared to existing techniques such as western blot and mass spectrometry and is aimed at helping scientists discover new therapeutic candidates earlier in the neurodegenerative disease cycle.

The new Revvity Revvity HTRF Phospho-Ubiquitin (Ser65) Cellular Kit joins Revvity's range of immunoassay kits and reagents for neuroscience applications across HTRF, Alpha, LANCE and DELFIA. For more information please refer to our scientific guide.

References

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Notes

CCCP solubility is limited in aqueous media, do not exceed 480 µM (0.1mg/mL) for intermediate dilutions in PBS or culture media. CCCP concentrations needed to activate mitophagy are much higher than those needed to depolarize mitochondria (<1 µM), usually CCCP >10 µM for several hours are required to elicit PINK1-induced mitophagy. The CCCP EC_{50} was shown to be linked to the fetal bovine serum (FBS) or bovine serum albumin (BSA) present in the cell culture media. Albumin contained in the FBS alters CCCP's ability to induce depolarization and subsequent PINK1-PRKN mitophagy. Soutar *et al. Autophagy* 2019,15(11), 2002–2011. FBS/BSA media concentration determines CCCP's ability to depolarize mitochondria and activate PINK1-PRKN mitophagy.

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