Development and validation of a high content-based assay to measure Tom20 loss in dopaminergic human neurons differentiated *in vitro*

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1 Introduction

Parkinson's disease (PD) pathogenesis has been linked to mitochondrial dysfunction and aberrant clearance of defective mitochondria through several lines of research. Particularly, targeting the autophagic destruction of mitochondria (mitophagy) has gained momentum as a therapeutic strategy for PD following the discovery of PD-linked mutations in genes such as Parkin and PINK1, which are key mitophagic regulators. Phenotypic assays to measure mitochondrial clearance in disease-relevant cellular backgrounds are therefore thought to represent powerful predictive tools to probe PD pathobiology and to identify potential PD therapeutics. Here, we describe the development of a high content-based assay to measure loss of the structural mitochondrial marker Tom20 (TOMM20) in differentiated ReNcell VM cells, which represent a replenishable source of human dopaminergic neurons.

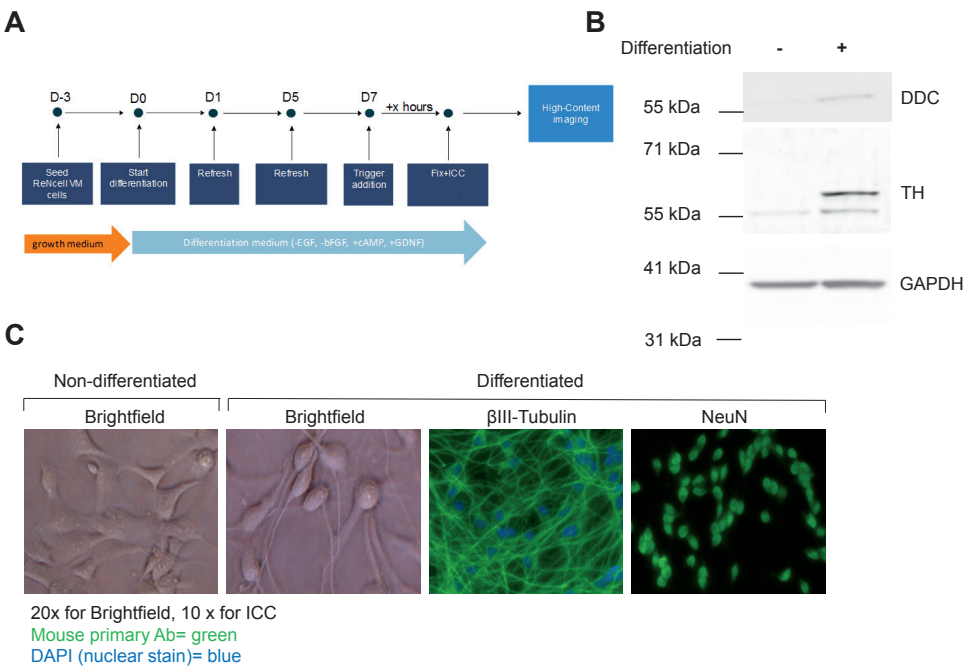


Fig. 1. Differentiation of ReNcell VM cells into neurons. ***(A)*** *General experimental outline.* ***(B)*** *Western blots demonstrating induction of the dopaminergic marker proteins DDC and TH (GAPDH as loading control).* ***(C)*** *Microscopic images demonstrating induction of neuronal phenotype and expression of the neuronal markers βIII-tubulin (middle right panel) and NeuN (rightmost panel) in differentiated ReNcell VM cells*

2 Tom20 immunocytochemistry

Tom20 expression in differentiated ReNcell VM cells (Fig. 1) was visualized using two antibodies in immunocytochemical stainings (Fig. 2). The Tom20 immunoreactivity pattern was consistent with mitochondrial localization and was time-dependently reduced by treatment with the mitochondrial uncoupler CCCP (Fig. 2). Tom20 co-localized with MitoTracker dye (Fig. 3), and Tom20 immunoreactivity was reduced by treatment with the well-known mitophagic trigger oligomycin/antimycin (Fig. 4). Taken together, these findings suggest that the Tom20 signal localizes to mitochondria and reflects mitochondrial abundance.

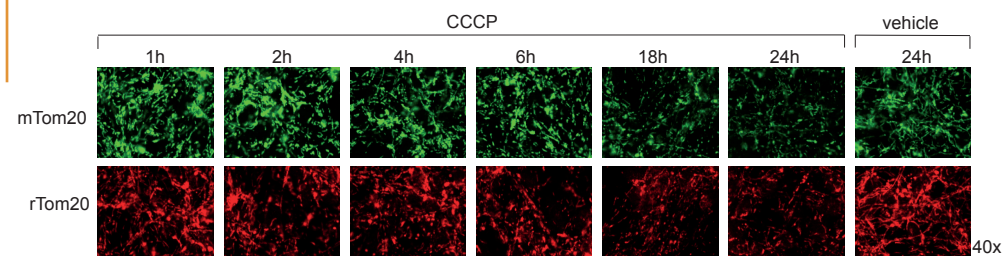


Fig. 2. Immunocytochemical staining of differentiated ReNcell VM cells using two antibodies directed against human Tom20. *The mitochondrial uncoupler CCCP (10 μM) or vehicle was added for the indicated treatment times followed by immunocytochemical analysis using mouse- (mTom20, Santa Cruz cat. # sc17764; in green) or rabbit anti-Tom20 (rTom20; Cell Signaling Technology cat. # 42406; in red)*

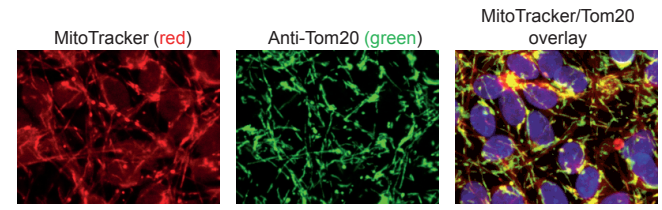


Fig. 3. Tom20 immunoreactivity co-localized with MitoTracker dye. *Differentiated ReNcell VM cells were treated with MitoTracker DeepRed FM dye (red; left panel) and subsequently fixed and immunostained using mouse anti-Tom20 (green; middle panel). The right panel represents an overlay of MitoTracker signal (in red), Tom20 immunoreactivity (in green) and nuclei (in blue).*

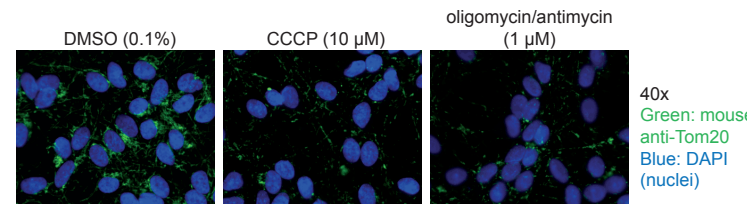


Fig. 4. Tom20 immunoreactivity was reduced by treatment with commonly used mitophagic triggers CCCP and oligomycin/antimycin. *Differentiated ReNcell VM cells were treated with vehicle (0.1% DMSO), 10 μM CCCP or 1 μM oligomycin/antimycin (O/A; equimolar concentrations) for 18 hrs followed by immunocytochemical staining using mouse anti-Tom20 (in green) and DAPI nuclear counterstain (in blue).*

3 High content-based analysis

To quantify Tom20 loss in the ReNcell VM model system, a high content-based algorithm was developed on the INCell 6000 imaging platform. The algorithm segments Tom20 immunoreactivity from immunocytochemical images and extracts fluorescence intensity as a quantitative measure of mitochondrial abundance (Fig. 5).

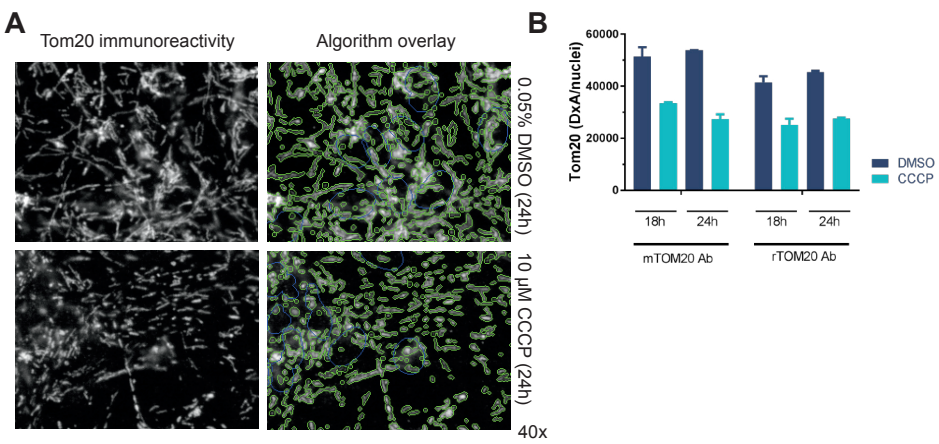
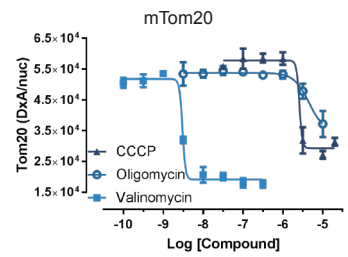


Fig. 5. High content-based quantification of Tom20 immunoreactivity ***(A)*** *Immunocytochemical image of Tom20 immunoreactivity in differentiated ReNcell VM cells (left panels) and the same image (right panels) with an overlay of the segmented Tom20 immunoreactivity (in green) and nuclei (in blue, as detected from the DAPI channel) detected by the high content-based analysis (HCA) algorithm. Cells were treated for 24 hrs with vehicle (upper panels) or 10 μM CCCP (lower panels).* ***(B)*** *Tom20 immunoreactivity quantified using the HCA-based algorithm (DxA/nuclei) following treatment with 10 μM CCCP for 18- or 24 hrs, and immunostaining with either mouse- or rabbit anti-Tom20 (mTom20 and rTom20, respectively). Statistical analysis: Student t-test;*
*ns = p > 0.05, * = p < 0.05, ** = p < 0.01*

4 Assay validation with tool compounds

The HCA-based algorithm was validated by quantification of Tom20 loss following treatment with ascending concentrations of well-known mitophagic triggers in 96-well plate format. Potencies in the Tom20 loss assay were consistent over two different antibodies used to detect Tom20 (Fig. 6).



Compound	Antibody	pIC_{50}
CCCP	mTom20	5.5
	rTom20	5.3
Valinomycin	mTom20	8.5
	rTom20	8.7
Oligomycin	mTom20	5.3
	rTom20	5.3

Fig. 6. Assay validation using prototypical mitophagy triggers. *Differentiated ReNcell VM cells were treated with increasing concentrations of CCCP, oligomycin or valinomycin for 18 hrs followed by immunocytochemistry using mouse anti-Tom20 antibody and HCA-based quantification of Tom20 immunoreactivity. pIC50s were calculated for Tom20 immunoreactivities derived from either mTom20- and rTom20-immunostained samples (see table).*

5 Conclusions

- **A high content-based Tom20 loss assay was developed to measure mitochondrial abundance in a dopaminergic neuronal *in vitro* model**
- **The assay represents a robust platform amenable to medium-to-high throughput screening of prospective PD therapeutics**
- **The assay can be modified to measure compound-mediated enhancement of mitophagy trigger-induced Tom20 loss**