

Assay Development and Validation of a High Content-based High-throughput Assay to Measure α -Synuclein Aggregation in Dopaminergic Human Neurons Differentiated *In Vitro*

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1 INTRODUCTION

Mutational analyses and post-mortem pathological studies have strongly implicated aggregation of the pre-synaptic protein α -synuclein in Parkinson's disease (PD). Despite interest from Pharma, widely accepted cellular models to probe α -synuclein biology with sufficient translational power have been lacking thus far. Many cellular models for α -synuclein aggregation developed to date possess poor resemblance to terminally differentiated, dopaminergic neurons or display a general lack of reproducibility or throughput to be compatible with drug discovery. Here, we describe the development of a high content analysis-based assay to measure α -synuclein aggregation in a terminally differentiated cell line of human mesencephalic origin, using an aggregate-selective anti- α -synuclein antibody termed MJFR14. This conformation-specific monoclonal antibody binds to aggregated α -synuclein with high affinity and specificity. The fully validated α -synuclein aggregation assay was miniaturized to 384-well format, automated and validated for high-throughput screening by testing a set of 1,000 small-molecule compounds.

2 RENCELL VM MODEL CHARACTERIZATION AND ADENOVIRAL TRANSDUCTION

ReNcell VM cells were selected as *in vitro* cellular model for the development of the α -synuclein aggregation assay based on their growth characteristics, differentiation potential into terminally differentiated neurons (Fig. 1) and amenability to adenoviral transduction (Fig. 2). Aggregation of human wild type α -synuclein, overexpressed through adenoviral delivery, was detected with the aggregate-specific anti- α -synuclein antibody MJFR14 using immunocytochemistry; total α -synuclein levels were detected with Syn205 (α/β -synuclein-specific antibody) (Fig. 2).

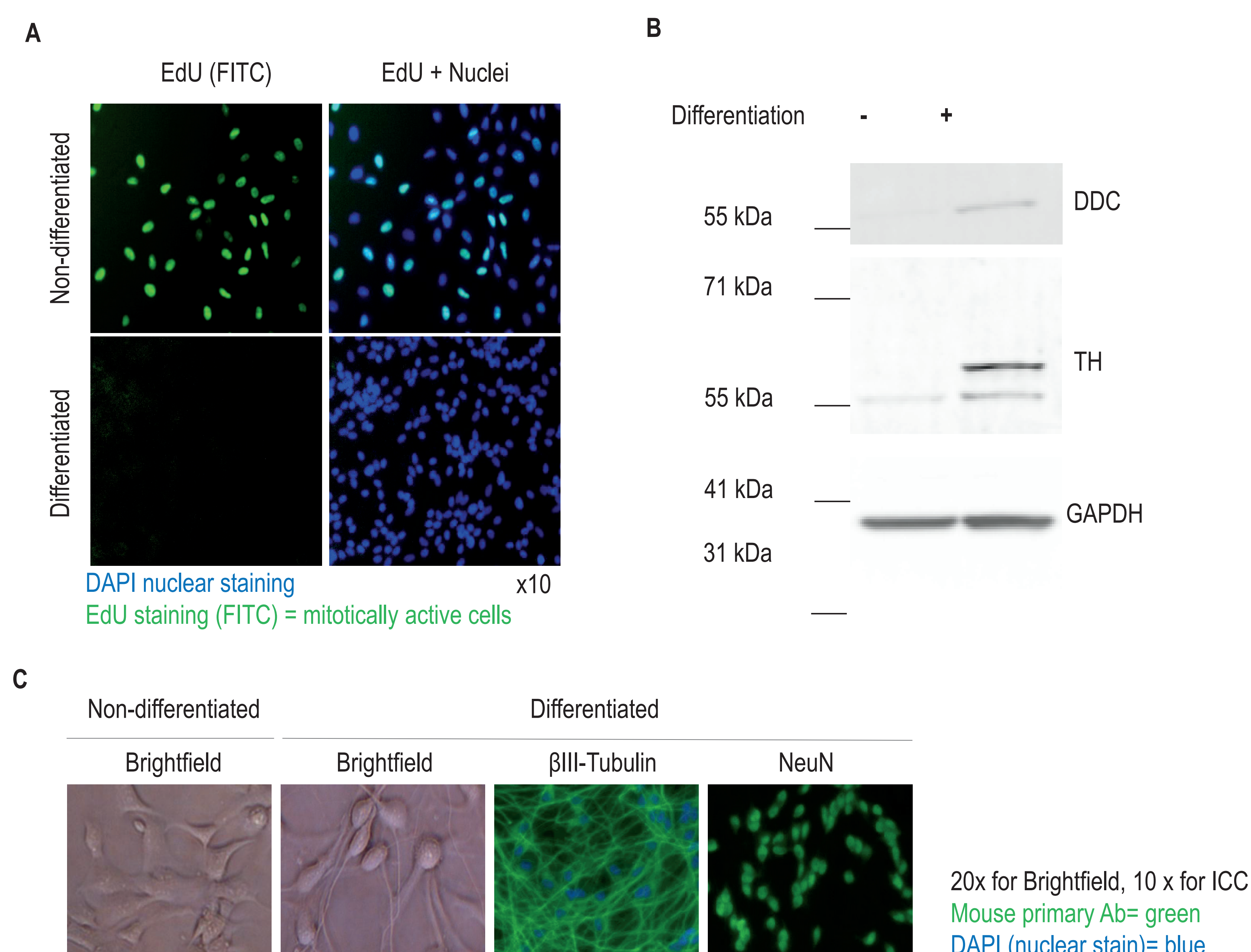


Fig. 1: ReNcell VM cells terminally differentiated into dopaminergic neurons. (A) EdU incorporation assay of non-differentiated (top panels) and differentiated (bottom panels) ReNcell VM cells. (B) Western blots of lysates isolated from non-differentiated (left lane) and differentiated (right lane) ReNcell VM cells probed with antibodies for the dopaminergic marker proteins DDC and TH and the loading control GAPDH. (C) Brightfield microscopic images of non-differentiated (leftmost panel) and differentiated (middle left panel) ReNcell VM cells and immunocytochemical detection of the neuronal markers β III-tubulin (middle right panel) and NeuN (rightmost panel) in differentiated ReNcell VM cells

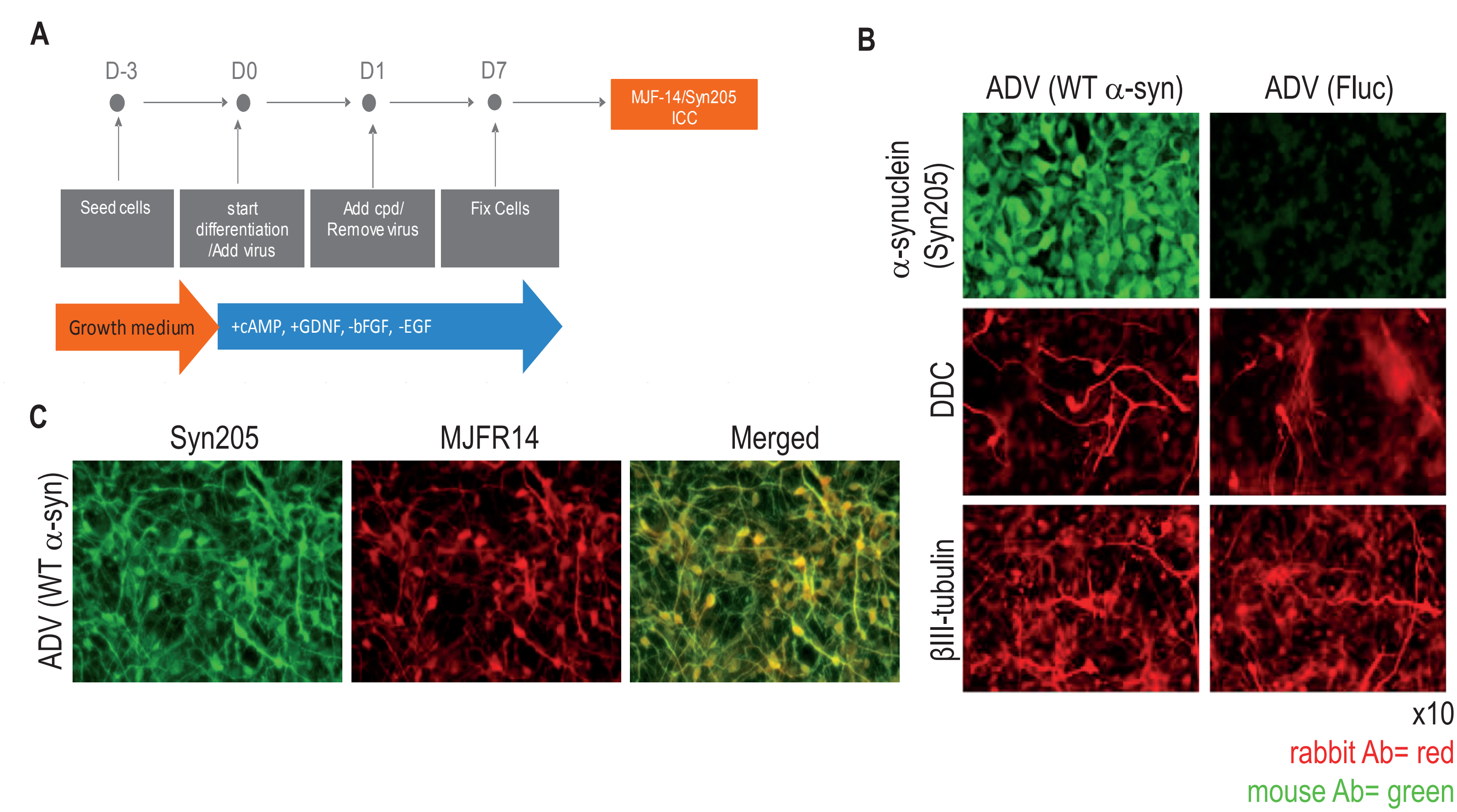


Fig. 2: Immunoreactivity of differentiated ReNcell VM cells immunostained with MJFR14 (aggregate-specific) and Syn205 (α/β -synuclein specific) antibodies following adenovirus-mediated α -synuclein overexpression. (A) Experimental outline of the α -synuclein aggregation assay. (B) Expression of total α -synuclein (Syn205; top panels), DDC (middle panels; dopaminergic marker) and β III-tubulin (bottom panels; neuronal marker) following transduction with proprietary adenoviruses (ADV) encoding wild type α -synuclein (WT α -syn; left panels) and firefly luciferase control protein (Fluc; right panels). (C) Immunocytochemical images of differentiated ReNcell VM cells transduced with adenovirus encoding wild type human α -synuclein and immunostained with Syn205 (left panel; α/β -synuclein) and MJFR14 (middle panel; aggregated α -synuclein). Right panel represents an overlay of Syn205- and MJFR14 images.

3 α -SYNUCLEIN AGGREGATION ASSAY DEVELOPMENT

Immunoreactivity towards aggregated (MJFR14) and total α -synuclein (Syn205) in adenovirally transduced ReNcell VM cells was quantified using high content-based segmentation of immunoreactive areas in immunocytochemical images (Fig. 3A), followed by quantification of immunofluorescent intensity (Fig. 3B). The small molecule PI3K inhibitor KU 0063794 was identified as a positive control compound for inhibition of MJFR14 immunoreactivity (Fig. 3B,C), most likely through activation of autophagy-mediated clearance of α -synuclein. Nuclei were quantified to control for compound cytotoxicity (fig. 3B).

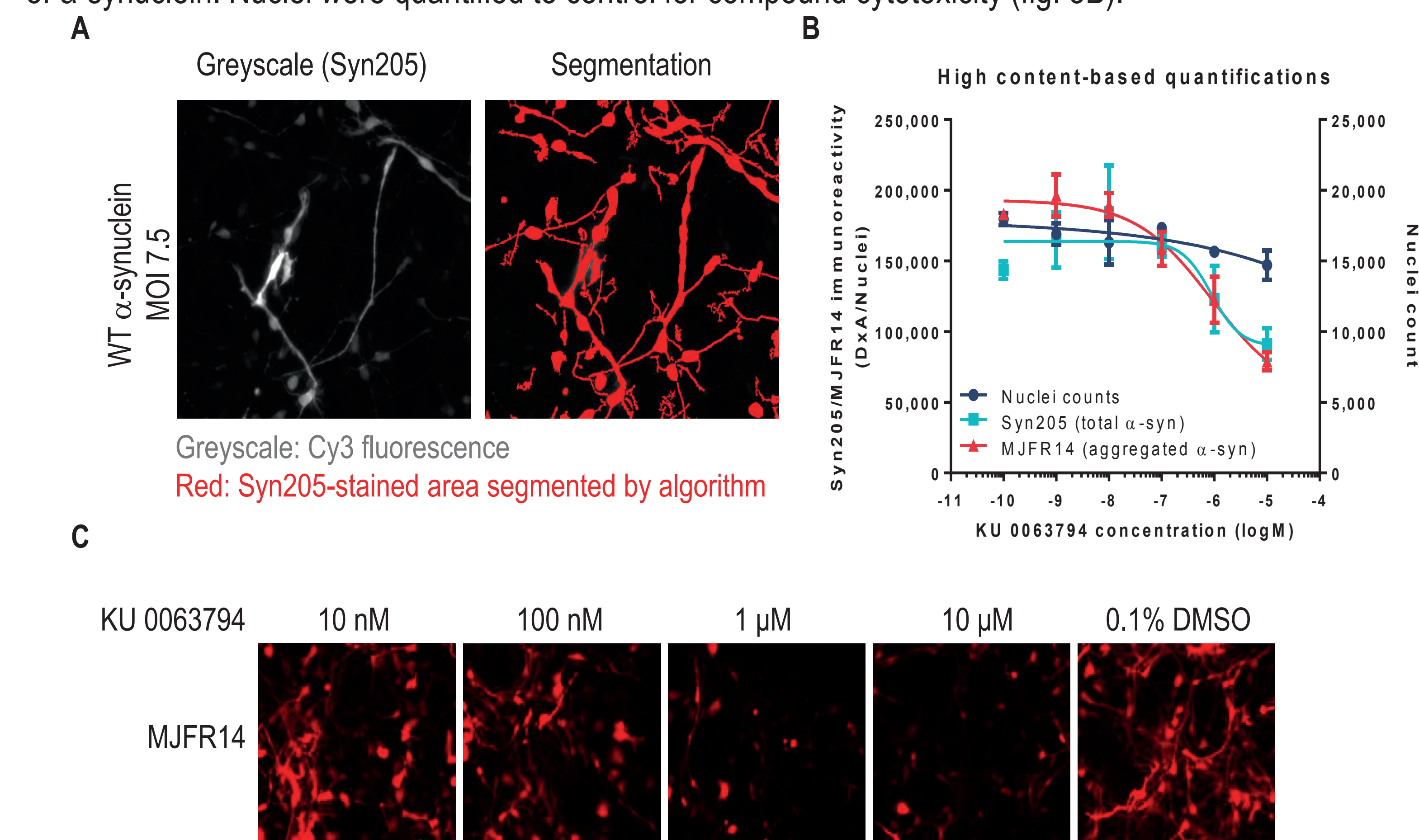


Fig. 3: High content-based quantification of Syn205- and MJFR14 immunoreactivity. (A) Segmentation of Syn205 immunoreactivity in α -synuclein-overexpressing, differentiated ReNcell VM cells. Left panel: immunocytochemical staining; right panel: same image with segmented area as red overlay. (B) Quantification of MJFR14/Syn205 immunoreactivity (Signal = DxA/nuclei where: D = immunofluorescent signal density and A = immunofluorescent signal area) following treatment with increasing concentrations of the PI3K inhibitor/autophagy inducer KU 0063794. Nuclei counts of KU 0063794-treated cells are quantified on the right Y-axis. (C) MJFR14 immunoreactivity following treatment with increasing concentrations of the PI3K inhibitor/autophagy inducer KU 0063794.

4 HIGH-THROUGHPUT SCREENING ASSAY VALIDATION – PILOT STUDY

The fully optimized α -synuclein aggregation assay was miniaturized to 384-well format and fully automated on robotic liquid dispensers. Subsequently, the assay was used to screen 1,000 small molecules in biological duplicate for their ability to selectively inhibit MJFR14 immunoreactivity (without affecting Syn205 immunoreactivity) (Fig. 4). KU 0063794 significantly and consistently reduced MJFR14 immunoreactivity on all plates tested (Fig. 4A) and coefficients of variation (%CV) for control conditions (DMSO, 10 μ M KU 0063794) were < 20% (fig. 4B). Concordance between biological duplicate samples was excellent (Fig. 4C; Spearman correlation coefficient > 0.5). Out of 1,000 compounds tested, 23 molecules specifically reduced MJFR14 immunoreactivity with minimal effect on Syn205 immunoreactivity (Fig. 4D).

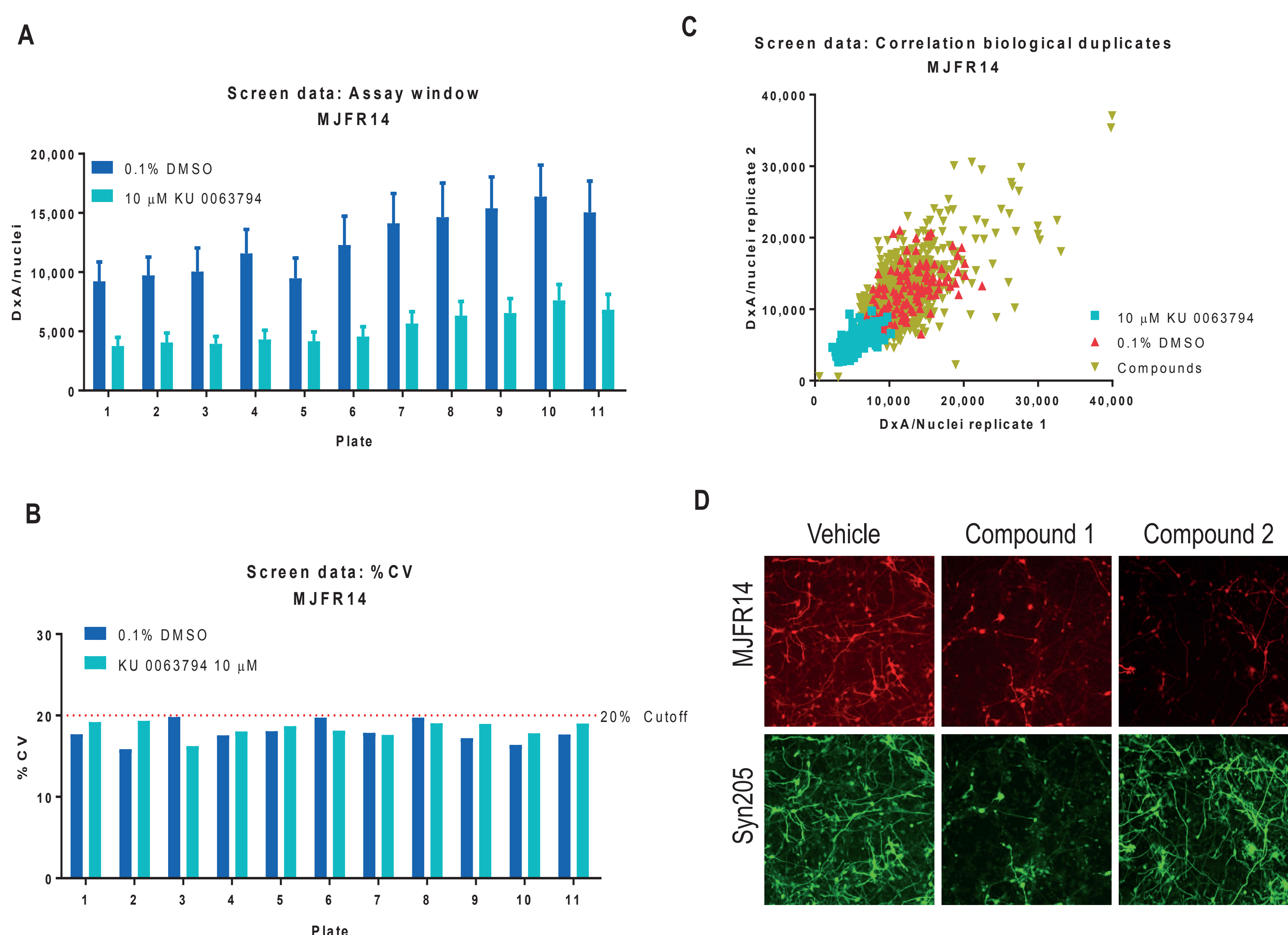


Fig. 4: Validation of the α -synuclein aggregation assay for high-throughput screening by screening 1,000 compounds in biological duplicate. (A,B) Plate-based comparison of (A) mean MJFR14 immunoreactivity and (B) coefficient of variation derived from up to 24 replicate control wells/plate treated with 10 μ M KU 0063794 or vehicle (0.1% DMSO). (C) Correlation plot displaying biological replicate data on the X- and Y-axis as a measure of data reproducibility. (D) Representative immunocytochemical images of MJFR14- (top panel) and Syn205-immunostained (bottom panel) ReNcell VM cells treated with DMSO (left panels), Compound 1, which equally reduced MJFR14- and Syn205 immunoreactivity (middle panels), and Compound 2, which specifically reduced immunoreactivity for MJFR14 (aggregated α -synuclein; right panels).

5 CONCLUSIONS

- The ReNcell VM model represents a replenishable source of human, terminally differentiated dopaminergic neurons that are amenable to adenoviral transduction using CRL's proprietary adenoviral platform.
- The fully automated 384-well α -synuclein aggregation assay enables high-throughput screening of small molecule inhibitors of α -synuclein aggregate formation as chemical starting points for disease-modifying therapeutics targeting Parkinson's Disease.