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In Vivo GCase Activity Assay

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Assay uses the substrate PFB-FDGluc (5-(Pentafluorobenzoylamino)Fluorescein Di-β-D-Glucopyranoside)from ThermoScientific (Cat# P11947). Substrate is supposed to be taken up by late endosomes and lysosomes only and fluoresces green when cleaved by lysosomal GCase. Described by Mazzulli et al., J Neurosci. 2016 Jul 20;36(29):7693-706.

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Measurement of GCase activity in lysosomes in live cells

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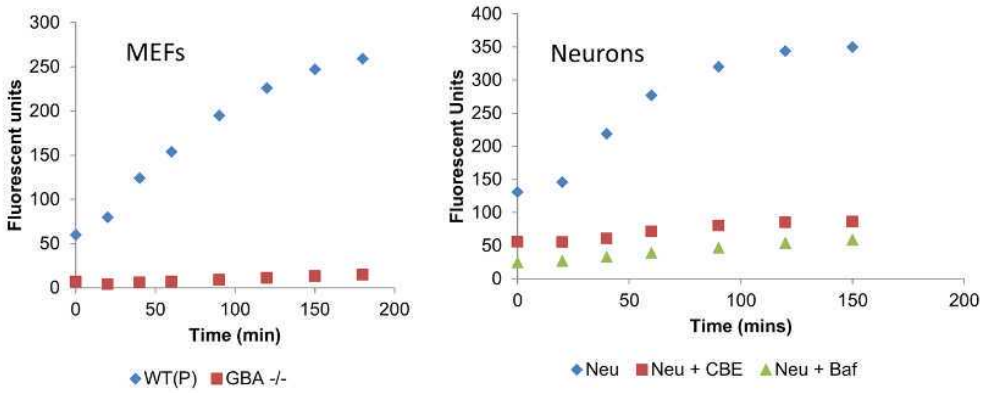
In my hands, no activity is detected in GBA -/- MEFs; activity in SH-SY5Y cells or primary neurons is abolished with CBE (10 µM,24h pretreatment) or bafilomycin A (100 nM, 2h pretreatment + present during substrate loading and assay). Human midbrain neurons differentiated from Wt/N370S iPSC exhibits 60% reduction of GCase activity.



I generally find it is linear for 60-90 minutes after substrate is washed away. It then starts to tail off either because substrate is exhausted, substrate leaks out of the cell or cells start to die (neurons don't look too healthy).

Method

2 Resuspend 5 mg of substrate in 100 ml methanol = 50 mg/ml stock. Do not expose to light. Store at -20 °C.
NB.

Methanol is toxicby ingestion, inhalation or exposure to skin. Resuspend substrate in fume hood wearing nitrile gloves and lab coat.

3 Cells cultured in 24 well plate. Should be >70% confluent to get robust signal. If too confluent, activity plateaus rapidly. As well as test wells (in triplicate) I have wells pretreated with 10 µM CBE for 24 hours.

4 Working substrate solution is 400 µg/ml (I don't get signal with lower concentrations). Prepare in OPTIMEM prewarmed to 37 °C: 250 µl/well. Therefore 2 ml of 50mg/ml stock substrate per 250 µl. I do not get results if I dilute substrate in phenol red free neurobasal media and use phenol red free OPTIMEM media during measurement of fluorescence.

5 Wash cells once carefully with prewarmed PBS.

6 Add 250 µl substrate (400 µg/ml in OPTIMEM) per well. Put in 37 °C incubator for 1 h.

7 Aspirate substrate. Wash cells three times with 250 µl PBS (37 °C).

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8 Add 250 µl OPTIMEM (37 °C). Measure t=0 minutes on plate reader: Ex, 488nm, Em, 520 nm. Return cells to incubator. Measure fluorescence every 20-30 minutes for up to 3 hours. Keep checking cells are alive with microscope.

9 At end of experiment carefully aspirate OPTIMEM. Lyse cells in wells with 200 µl 1% TX-100 in PBS on ice for 15 minutes. Pellet debris at 17,000 xg, 10 minutes, 4°C. Measure protein concentration in supernatant with BCA assay.

Calculation

10 Calculate total protein per well: protein concentration (mg/ml) x 0.2 = mg protein per well

For each well at each time point (including CBE treatment): Divide Fluorescent units by protein per well (mg) = Fluorescent units/mg protein

For GCase rate: subtract CBE fluorescent units/mg protein from test sample fluorescent units/mg protein.

Example at t=0 mins.

Sample 1 = 85 fluorescent units; protein in well = 0.25 mg

Sample 1 + CBE= 8 fluorescent units; protein in well = 0.24 mg

Therefore: (85/0.25)-(8/0.24) = 307 fluorescent units/mg protein

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