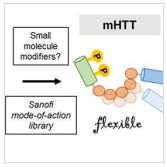

Mar 02, 2021

Time-resolved FRET in 384-well plate format to identify small molecular modifiers of mutant Huntingtin conformational inflexibility

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1 Works for me dx.doi.org/10.17504/protocols.io.bsw8nfhw

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SUBMIT TO PLOS ONE

ABSTRACT

BACKGROUND

Huntingtin (HTT), if mutated, is the key driver of the neurodegenerative Huntington's disease (HD). Wild-type HTT (wtHTT) and excessive poly-glutamine containing mutant HTT (mHTT) differ in the intramolecular flexibility of their N-terminal region. The loss of flexibility and conformational change in mHTT is thought to drive protein aggregation and to decrease protein-protein interactions with other critical neuronal proteins. Decreased conformational flexibility of mHTT therefore most likely results in neurodegeneration. It has been demonstrated that post-translational modifications (PTMs), such as N-terminal amino acid phosphorylation, can rescue mHTT inflexibility.

METHODOLOGY

Time-resolved FRET (TR-FRET) using the two HTT N-terminus-specific antibodies 2B7 (donor, labelled with terbium cryptate (Tb)) and MW1 (acceptor, labelled with D2) can detect intramolecular conformational changes of HTT. This is especially evident when the protein is subject to temperature changes: Flexible wtHTT shows a high degree of flexibility and low FRET signal at 20°C while at 4°C intramolecular flexibility is reduced and the FRET signal increases. Therefore, the 4°C/20°C FRET signal ratio is > 1 for wtHTT. mHTT is locked in a rigid state and the FRET signals at 20°C and 4°C are more similar, although not equal. As a result, the 4°C/20°C FRET signal ratio is close to 1. Since mHTT inflexibility is influenced by N-terminal PTMs and TR-FRET can measure mHTT conformation, small molecule libraries can be screened for their effects on mHTT conformational rescue. A KNIME workflow is provided to perform plate data aggregation.

ANTICIPATED OUTCOME

Compounds that restore mHTT conformational flexibility should lead to a 4°C/20°C FRET signal ratio of > 1. This effect could be either due to a direct binding of a small molecule to mtHTT or via an indirect influence on PTMs i.e. the stimulation of a kinase or inhibition of a phosphatase. Compounds with no effect on mHTT flexibility should not influence the 4°C and 20°C FRET signals and the ratio should remain close to 1.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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ATTACHMENTS

KNIME-Workflow_Plate_data_aggregation.knwf

DOI

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KEYWORDS

Huntington's disease, Huntingtin, time-resolved FRET, screen

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MATERIALS TEXT

- GM04691 fibroblasts (affected donor, passage #10), Provided by Evotec, Storage: Liquid nitrogen
- GM04729 fibroblasts (healthy donor, passage #9), Provided by Evotec, Storage: Liquid nitrogen
- 384-well assay plate, white, flat bottom, cell culture treated, Greiner 781080, Lot: E19043HU
- DMEM + Glutamax, Gibco 105566-016, Lot: 2026753, Storage: +4°C
- FBS, Gibco 16000-044, Lot: ?, Storage: -20°C
- Pen/Strep 100X, Gibco 15140-122, Lot: 209152, Storage: -20°C, Stock Concentration: 10000 U/ml
- PBS-/-, Gibco 14190-094, Lot: 2026787, Storage: +4°C
- Trypsin 0.25%, Gibco 25200-056, Lot: 2026991, Storage: +4°C
- Trizma base, Sigma T1503, Lot: SCBV6926, Storage: +4°C
- Trizma HCl, Sigma T3253, Lot: SCBX6980, Storage: +4°C
- Igepal (CA-630), Sigma 18896-50mL, Lot: MKCC9036, Storage: +4°C
- Bovine Serum Albumine (BSA), Sigma A7030-500G, Lot: SCBS2014V, Storage: +4°C
- Protease & phosphatase inhibitor cocktail, Thermo Scientific 1861281, Lot: TH271623A, Storage: +4°C
- DMSO, Sigma D5879-500ML, Lot: 056K0173, Storage: RT
- Antibody MW1, DHSB AB_528290, Lot: 10ea9/1/17-1mg/ml, Storage: -20°C, purified and labelled with D2 by Cisbio, Lot 190329, Storage: -80°C
- Antibody 2B7-Tb (CHDI Foundation, purified by Sanofi Biologicals), purified and labelled with Terbium cryptate by Cisbio, Lot 190513, Storage: -80°C
- Compound library of choice, ideally pre-diluted in DMSO. This protocol has been optimized for screening of up to 4,000 compounds.

1 Seed 10,000 cells/well (50 ul, GM04691 fibroblasts) in DMEM + 15% FBS + PenStrep in Greiner 384 well plates according to the anticipated plating scheme. Do not seed cells in the left-most column 1. Later, this column will serve to calculate the FRET background signal. When seeding multiple plates, use a Multidrop cell dispenser and centrifuge plates briefly to ensure all liquid is at the bottom of the well.

1.1 **Reagent preparation** - Growth medium

A	B	C
Reagent	**Volume**	**Final concentration**
DMEM+Glutamax	500 ml	N/A
FBS (100%)	89 ml	15%
Penicillin / Streptomycin 100X	5.9 ml	1X

2 Incubate plates for 24 hours at 37°C and 5% CO2.

3 If possible, use robotic liquid handling for the next steps. Treat cells with compounds or DMSO for 24 hours (3 uM of each library compound, 3 ul per well). 3 plates were treated with DMSO only and 6 plates were treated with library compounds in duplicate.

4 Aspirate 40 ul medium (rest : 10 ul).

5 Lyse cells with 10 ul TR-FRET lysis buffer (2X concentrated).

5.1 **Reagent preparation** - TR-FRET lysis buffer (100 ml total)

A	B	C
Reagent	**Volume**	**Final concentration**
100mM Tris, pH7.4	10 ml	10 mM
5M NaCl	3 ml	150 mM
H2O	85 ml	N/A
Protease/phosphatase inhib.	2 ml (add just before use)	2%
Igepal	0.6 ml (add just before use)	0.6%

6 Incubate plates for 1 hour at room temperature.

7 

Add 10 ul of 2B7-Tb/MW1-D2 in TR-FRET antibody buffer (3X concentrated) to reach the desired concentration in the wells. See the antibody dilution table for details.

7.1 **Reagent preparation** - TR-FRET antibody buffer (65 ml total)

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A	B	C
Reagent	**Volume**	**Final concentration**
100mM Tris, pH7.4	6.5 ml	10 mM
5M NaCl	1.95 ml	150 mM
H2O	56.55 ml	N/A
Igepal	0.195 ml (add just before use)	0.25%
BSA (add just before use, prepared as 10X for ease of use)	0.065 g (add just before use)	0.1%
Antibodies	Add just before use (see table below)	

7.2 **Reagent preparation** - Dilution of antibodies in TR-FRET antibody buffer

A	B	C	D	E	F	G
Antibody	**Stock antibody concentration (ng/ul)**	**Desired antibody concentration (3X) (ng/ul)**	**Total required volume (ul)**	**Amount of stock to add to required volume (ul)**	**Obtained concentration**	**Final concentration in well (10ul + 20ul)**
2B7-Tb	245	0.075	65,000	19.90	3X	1X
MW1-D2	753	0.75	65,000	64.74	3X	1X

8

To prevent evaporation, seal the plates with Thermoplast adherent foil and incubate at 20°C overnight for 16 hours. In order to prevent too low FRET efficiency results, under no circumstances should the temperature be higher than 22°C during incubation.

9 Read plates on a PheraStar plate reader (200 flashes, 60 – 460 us integration window, focal height: 8.9 mm). Export the data as a .csv file.

10 Incubate the plates for 2 hours at 4°C.

11 Repeat the reading on the PheraStar plate reader using identical settings. Export the data as a .csv file.

12 Data analysis step 1: Aggregate the multiple results files (one for each temperature per plate). The provided KNIME workflow can be used to perform this task. Depending on the experimental setup, this KNIME workflow can be easily

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adapted to perform all the calculations outlined below without any coding experience.

13 Data analysis step 2: Calculate the 665nm/620nm ratio (FRET efficiency) for both temperatures (4°C & 20°C.) for all individual wells.

14 Data analysis step 3: Calculate the mean 4°C/20°C ratio for all cells which did not contain cells in order to determine the background FRET signal.

15 Data analysis step 4: Subtract the mean 4°C/20°C background FRET signal from all DMSO- and compound-containing wells.

16 Data analysis step 5 to identify active compounds: For all DMSO-only containing wells, calculate the median and standard deviation of the 4°C/20°C background-corrected FRET signal. Active compounds should have a 4°C/20°C background-corrected FRET signal which is higher than the DMSO median + three times the DMSO standard deviation.

17 Ideally, the screen is repeated on a different day. Compounds that are identified twice as active compound are considered as hit compounds.

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