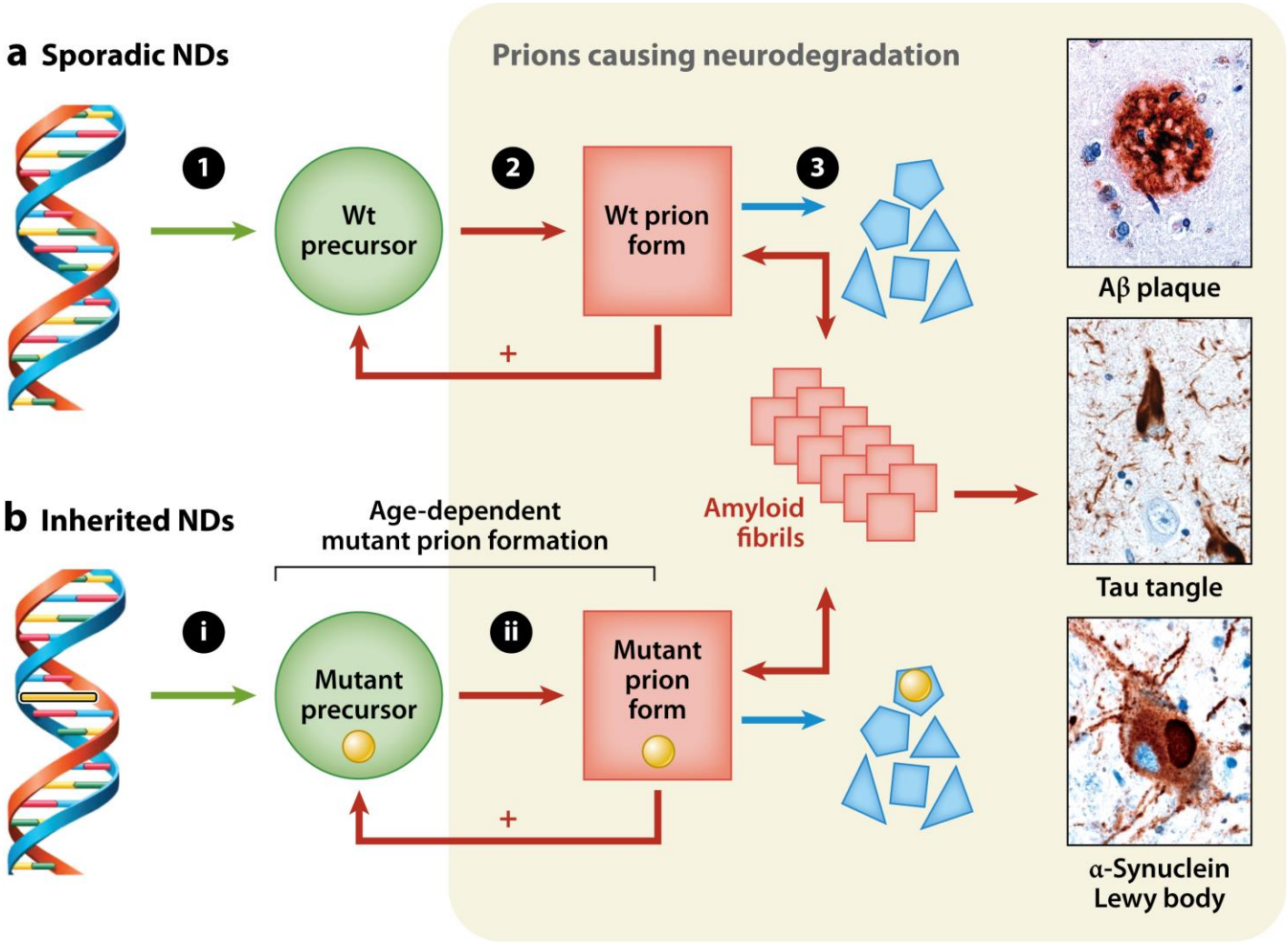
High Throughput Screening For Prion Therapeutics

Joel Gever

April 4, 2014

Emerging data suggests most important neurodegenerative disorders are prion diseases



Prusiner SB. 2013.
Annu. Rev. Genet. 47:601–23

Selection of cell type is an important decision for phenotypic screens

- PrP^{Sc}:
  - No infected human cell lines have been developed to date
  - N2a mouse neuroblastoma infected with RML mouse adapted-scrapies
  - CAD5 infected with RML and other prion strains (D. Berry)

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- PrP^{C}:
  - T98G human glioblastoma
  - IMR32 human neuroblastoma
  - N2a mouse neuroblastoma

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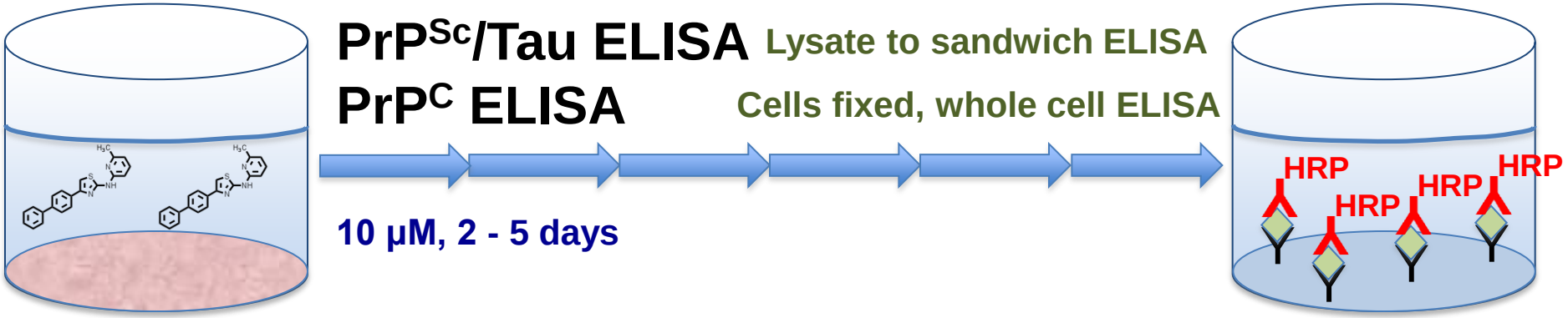
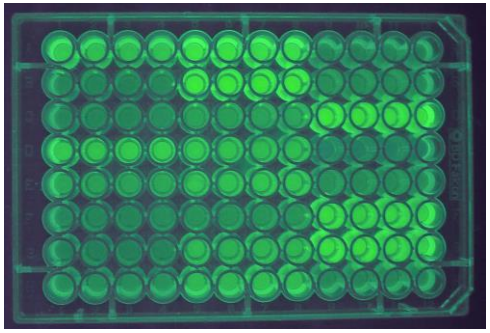
- PrP^{C}:
  - T98G human glioblastoma
  - IMR32 human neuroblastoma
  - N2a mouse neuroblastoma
- Tau:
  - T98G human glioblastoma
  - IMR32 and SH-SY5Y human neuroblastoma
  - HEK293-RD-YFP (Kfoury, JBC, 2012)
  - Differentiated neural progenitor cells derived from human induced pluripotent stem cells (Haggarty, Biol. Psychiatry, 2013)

HTS at IND relied on protein reduction ELISAs (historically)





"Glowing well" assay requires separate cell viability measurement to eliminate false positives due to cell death

- **Calcein** (intact membrane and functioning esterases)
- **Cell TiterGlo** (total ATP)

IND chemical library: ≈ 130,000 compounds

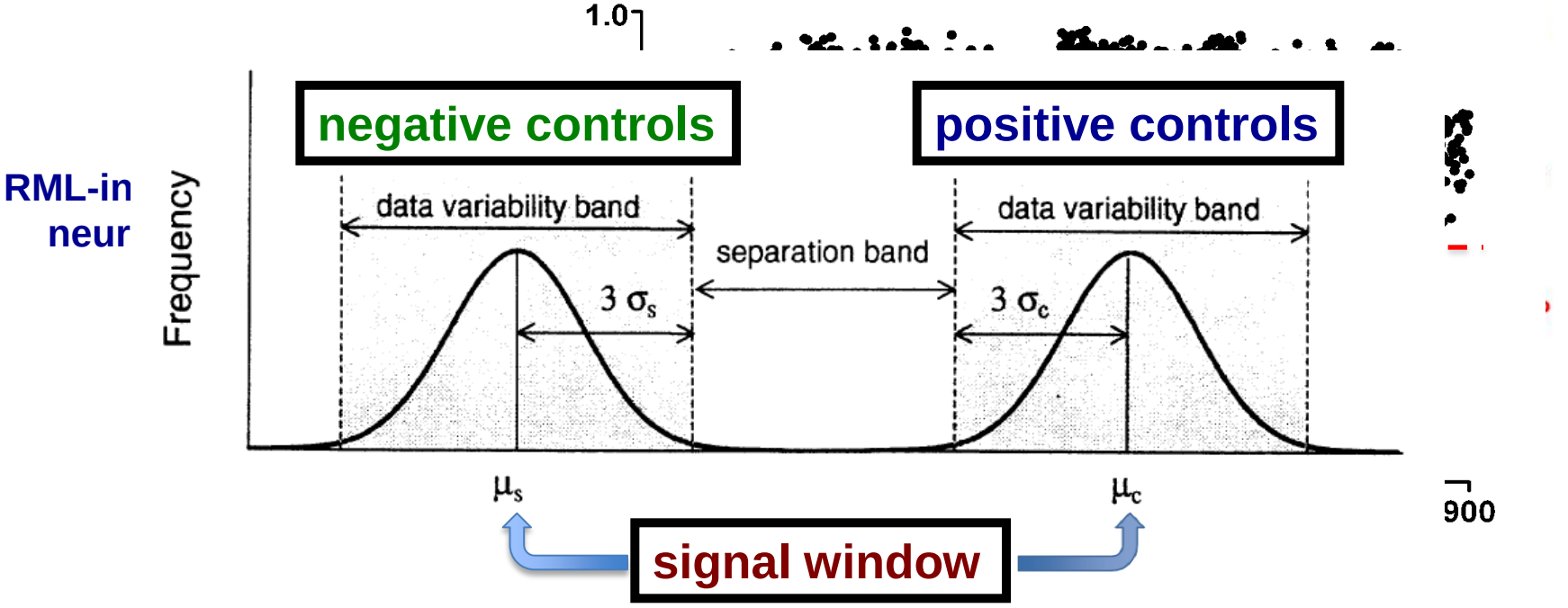
IND database has 159,693 registered compounds and consists primarily of:

Library	# of Compounds	ChEMBL Target[1]
Chembridge (CB-1)	23,759	75%
Chembridge CNS-Set (CB-2)	39,840	70%
SPECS (via SMDC)	30,104	70%
ChemDiv Diversity	21,995	n.d.
Life Chemicals	30,400	n.d.
Broad Institute	9,513	n.d.
Johns Hopkins Clinical Compounds	1,420	n.d.
Analogs and other small collections[2]	≈ 2000	n.d.

[1] % of 1652 targets with ≥ 10 compounds predicted to hit (John Irwin, UCSF)

[2] University of Kansas, UCLA, Microsource, analog by catalog, synthesized molecules

ScN2a PrP^{Sc} ELISA

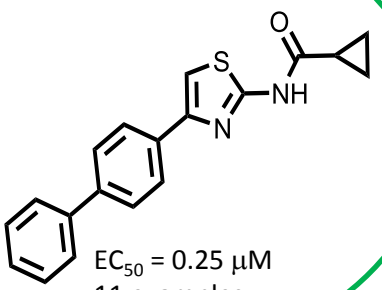


- One common criterion of a "good" assay
  - $Z' = 1 - [(3*SD_{pos} + 3*SD_{neg})/|(mean_{pos} - mean_{neg})|]$
  - **Z' ideally should be ≥ 0.5**

Several promising leads emerged from the PrP^{Sc} HTS campaign

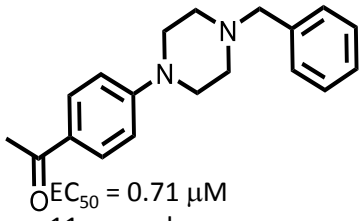
- 66,496 compounds screened in dividing cells[1]
  - 14 structural classes found by clustering analysis
  - Aminothiazoles were among the most potent and a focus of early optimization efforts[2]

Aminothiazoles



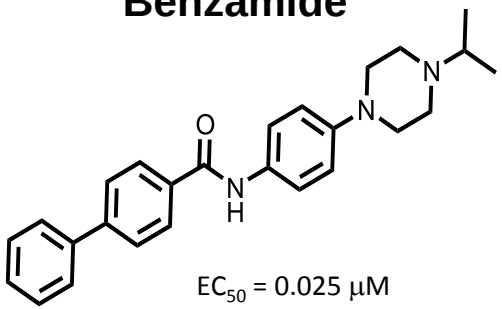
EC_{50} = 0.25 μM
11 examples

Piperazine



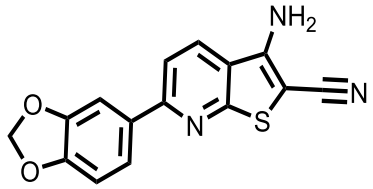
EC_{50} = 0.71 μM
11 examples

Benzamide



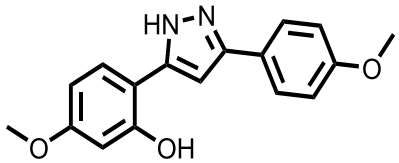
EC_{50} = 0.025 μM
95 examples

Thienopyridine



EC_{50} = 0.98 μM
54 examples

Pyrazole

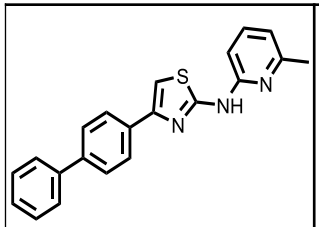
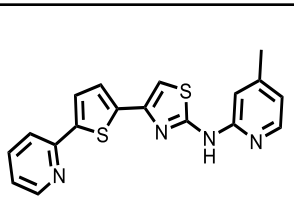
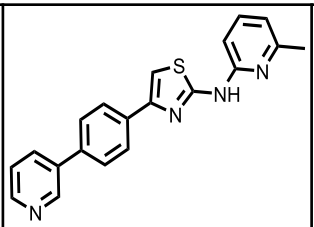
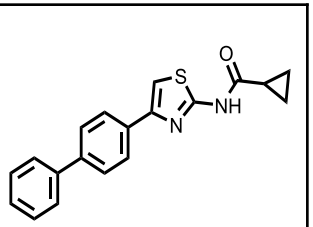


EC_{50} = 0.07 μM
6 examples

1. Silber, B.M., *et al*. (2013). Bioorg Med Chem
2. Ghaemmaghami, S., *et al*. (2010). J Virol

Optimization of the aminothiazoles

- Potency was optimized while maintaining and improving ADME properties[3,4]

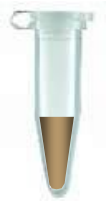
	 IND-24	 IND-81	 IND-114338	 IND-30410
EC_{50} ScN2a (μM)	1.29	1.95	0.068	0.248
Mouse liver microsomes $T_{1/2}$ (min)	>60	19	>60	>60
Brain AUC_{last}/EC_{50} (10mg/kg PO)	22.6	2.3	160	75

- Compounds in the series have excellent brain penetration, reasonable bioavailability (IND24 = 40%), and metabolic stability, and show no apparent toxic effects in long term dosing in mice[5]

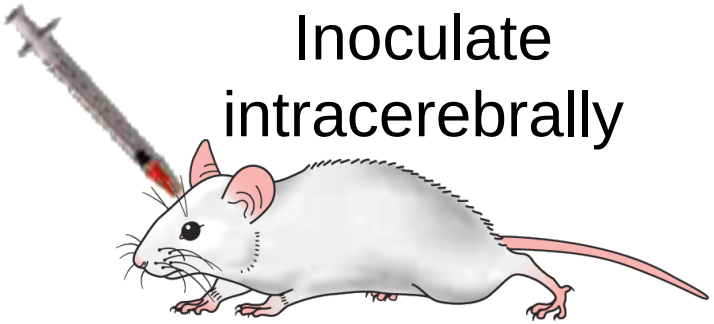
3. Gallardo-Godoy, A., *et al*. (2011). J Med Chem
4. Li, Z., *et al.* (2013). Chem Med Chem
5. Silber, B.M., *et al.* (2013). Pharm Res

Animal models for prion disease

Prion-infected mouse brain

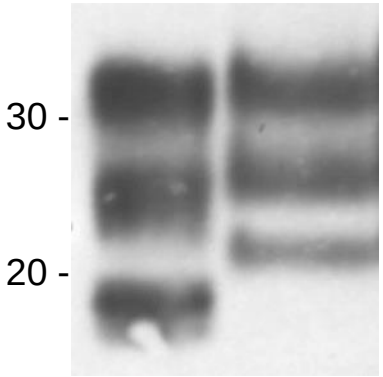


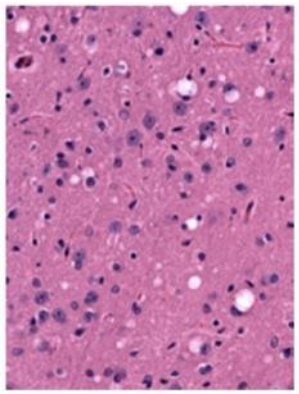
Homogenize



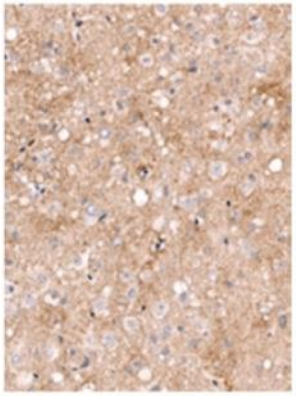
Inoculate intracerebrally

Monitor for onset of clinical signs

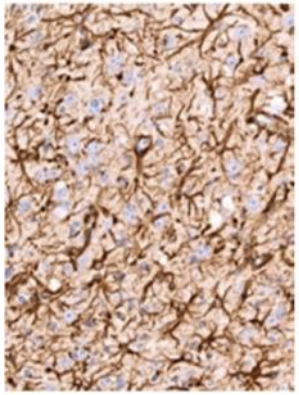




H & E



PrP



GFAP

Kurt Giles, IND, UCSF

Effects of IND24 treatment in Tg mouse models of prion disease

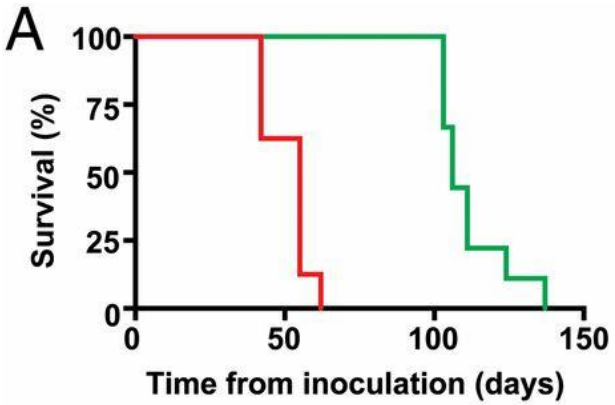
Tg1053 (mousePrP)

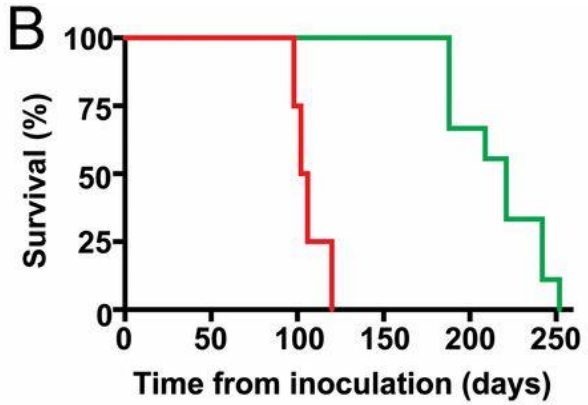
Tg1014 (mouse/humanPrP)

IND24

Control

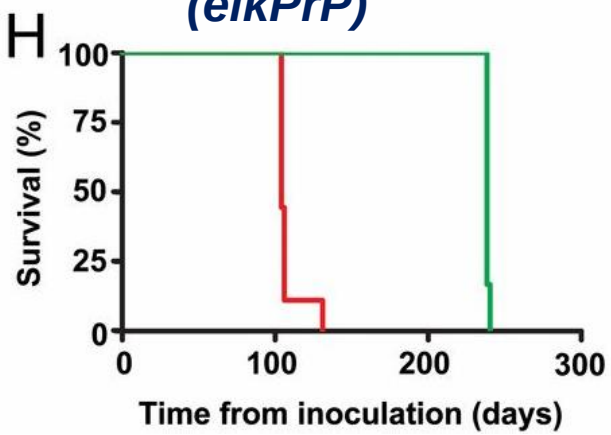
RML (mouse PrP^{Sc})





Tg12584 (elkPrP)

CWD (elk PrP^{Sc})



Berry D B et al. PNAS 2013;110:E4160-E4169

Effects of IND24 treatment in Tg mouse models of prion disease

Tg1014 (mouse/human PrP^C)

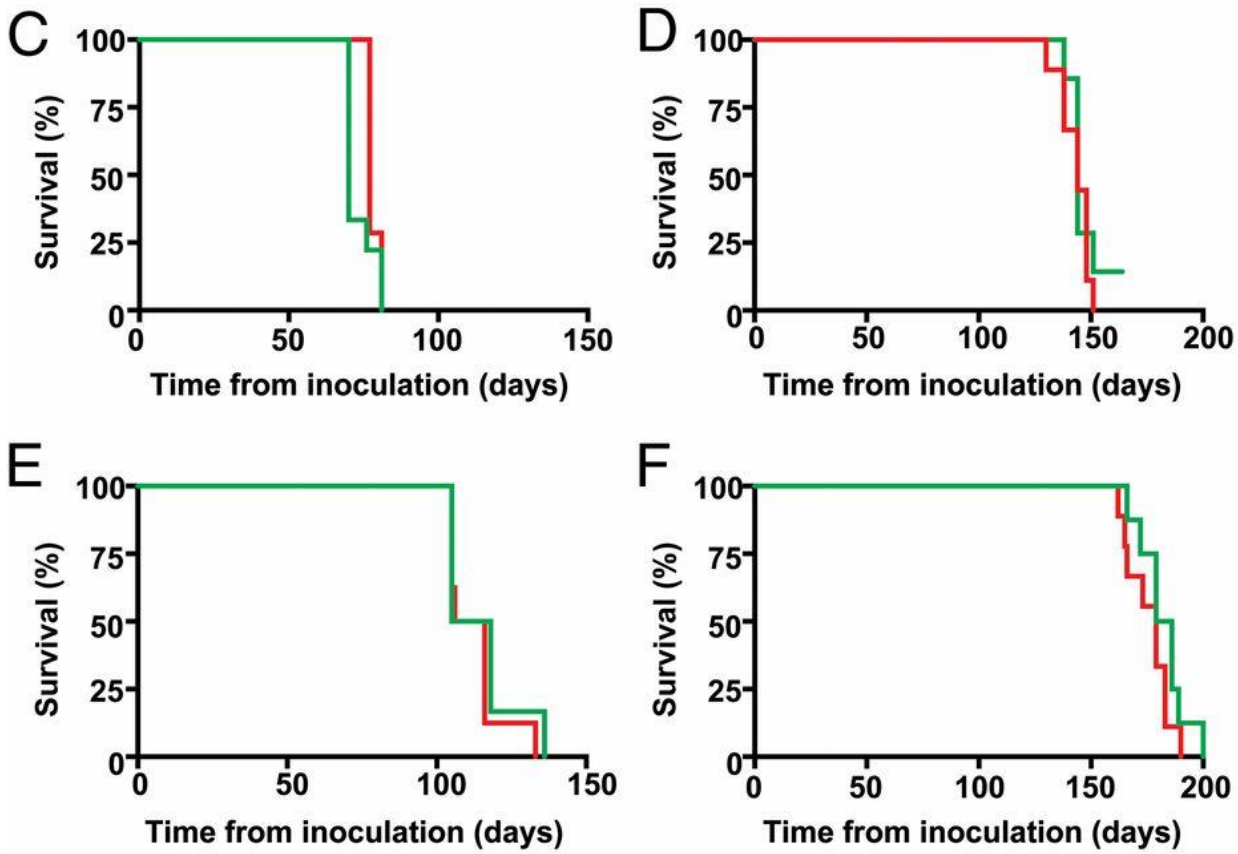
Tg2669 (human PrP^C)

IND24

Control

sCJD (human PrP^{Sc})

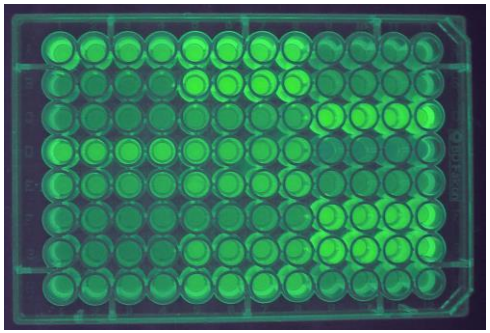
sCJD (human PrP^{Sc}) 1:1000 dilution



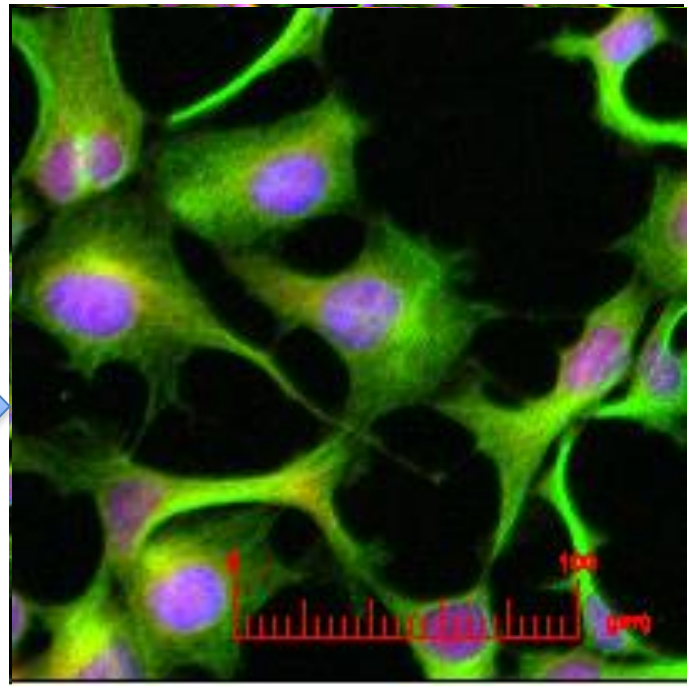
Berry D B et al. PNAS 2013;110:E4160-E4169

Assay format has shifted primarily to high content analysis (HCA)

ELISAs are convenient because all you need is a plate reader



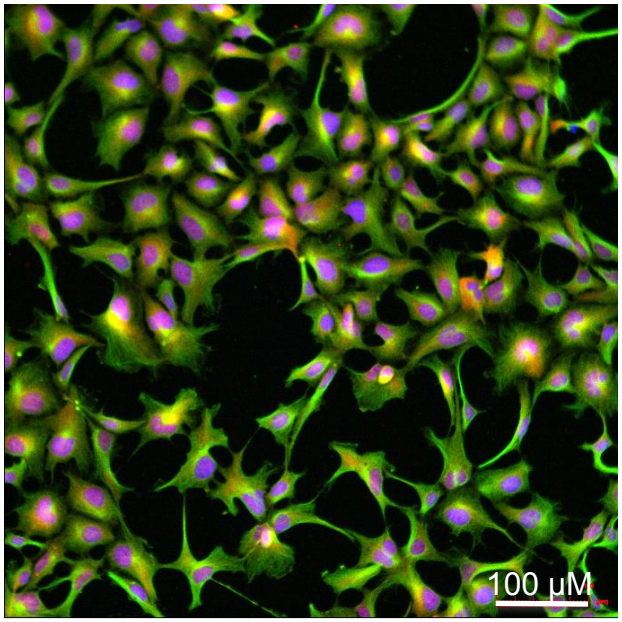
But why settle for glowing wells when you can have this?



Undifferentiated neural progenitor cells (NPC)
Nucleus (blue), tubulin (green) and tau (red)
(kindly provided by Haggarty Lab, Harvard University)

HCA is superior to ELISAs in most ways

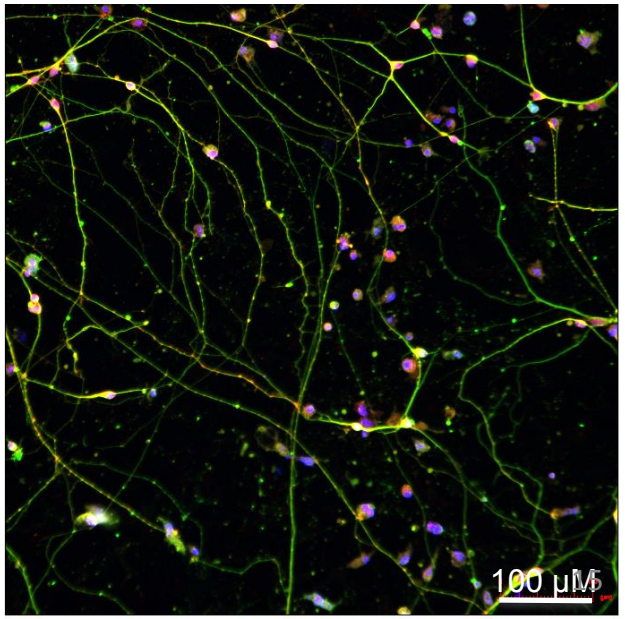
- Orders of magnitude more information
  - **Thousands of readouts in each well**
  - **Can measure labeled macromolecules in subcellular regions**
  - **Can measure up to 4 wavelengths simultaneously**
  - **Measurements only in cells that are present (cell viability assay not required as in ELISA)**
- Can use cells available in small quantities



NPCs differentiated

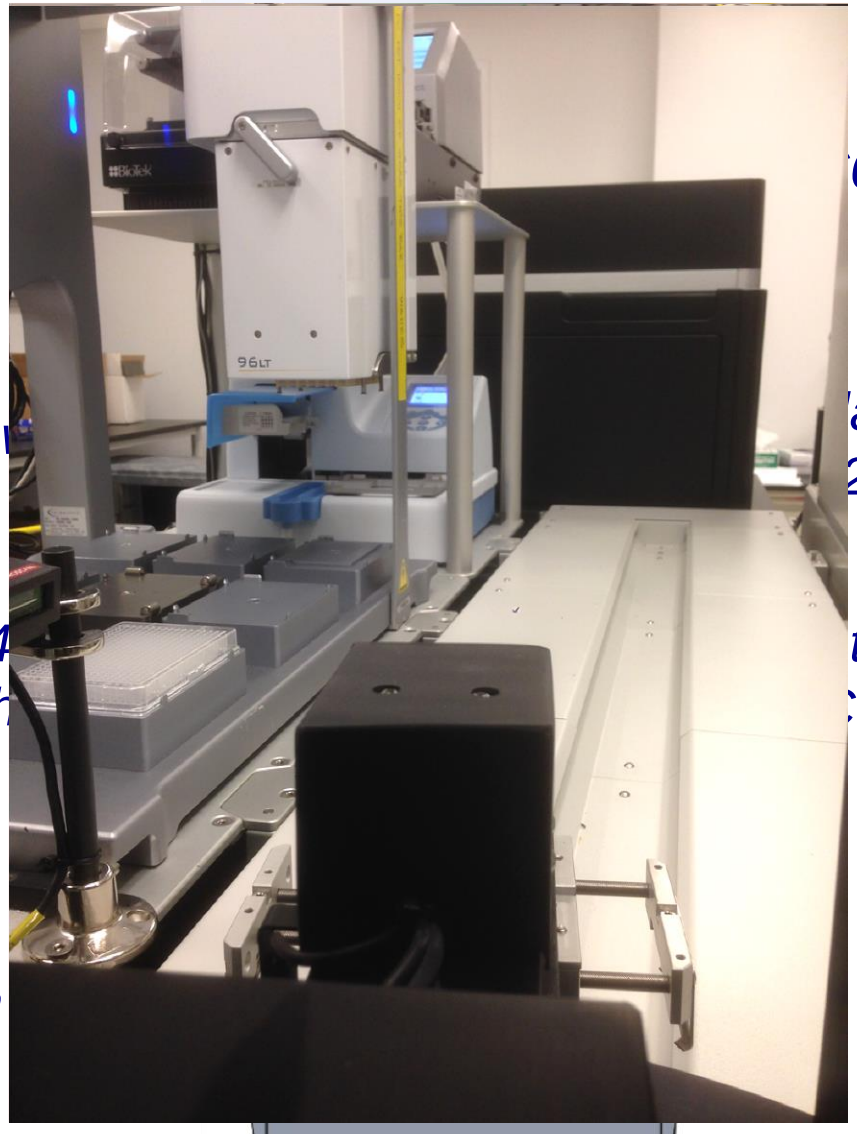
for 21 days

Nuclei (blue)
Tau (red)
Tubulin (green)



Automation enables high throughput for time consuming assays

1. 30 – 60 minutes plate read time by HCA; 24/7 screening will more than triple throughput
2. Semi-automation of plate preparation may increase throughput
3. With scheduling software, can weave activities together (e.g. hitpicking)



000

Plate

lates,

20 ° C

96/384

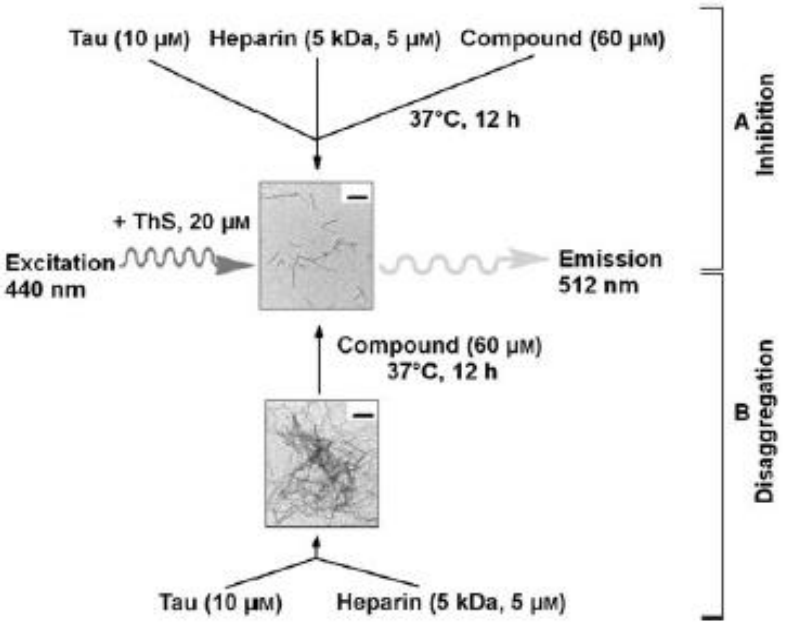
liquid h

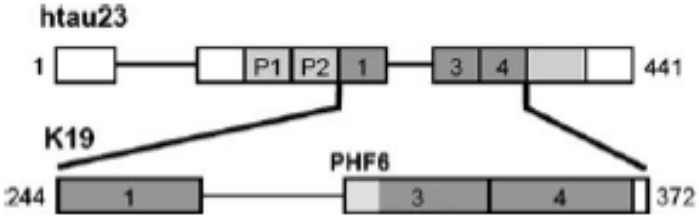
tes,

Tecan

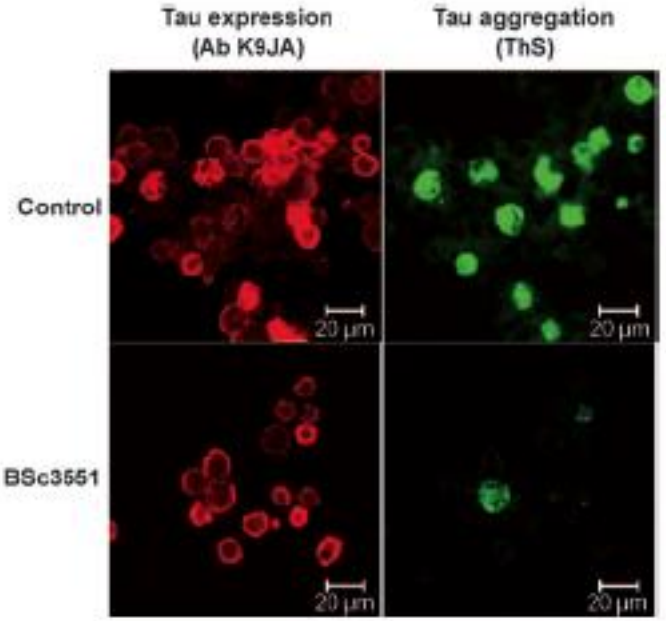
Tau aggregation may be a promising approach

- Published tau aggregation HTS campaigns have utilized protein fibrillization assays (cell-free) to identify hits (e.g. K19 + heparin)



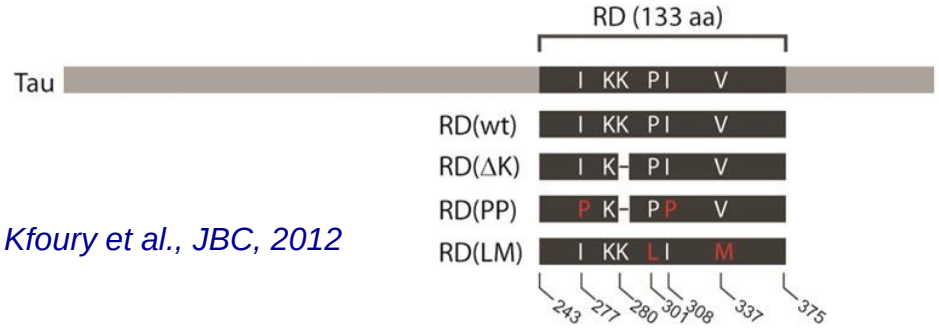


- Confirmation by Thioflavin S



Bulic et al., 2009

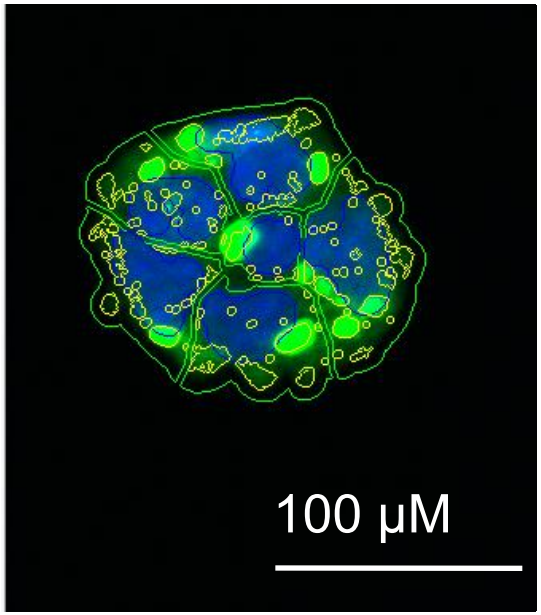
Tau aggregation using HEK-RD(LM)-YFP

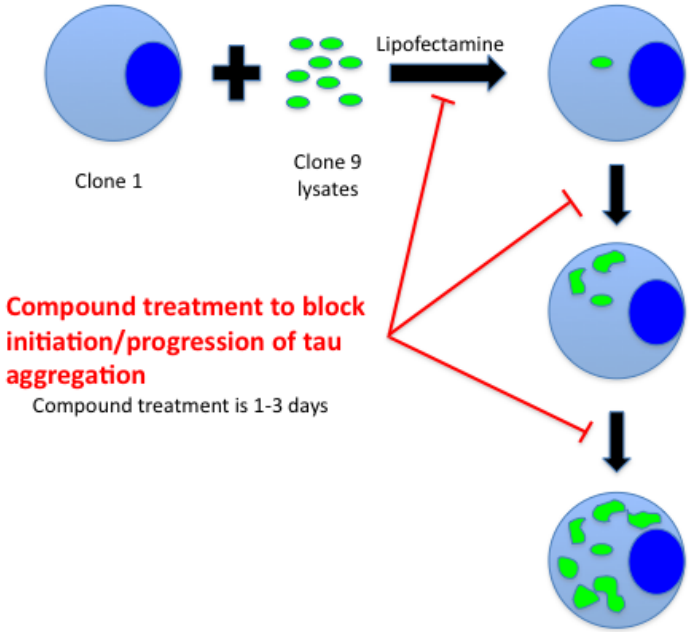


Kfoury et al., JBC, 2012

- HEK293 cells transfected with repeat domain containing double mutation (P301L and V337M)
- Conjugation to YFP enables live cell imaging
- Two subclones (clone 9 and clone 10) are phenotypically distinct and stably express tau aggregates

HEK-RD(LM)-YFP clone 9



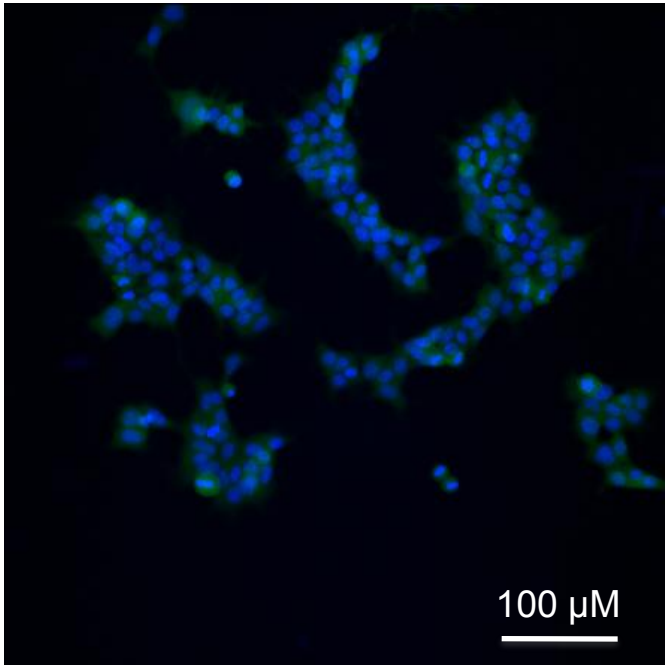


Cells kindly provided by Marc Diamond, Washington University

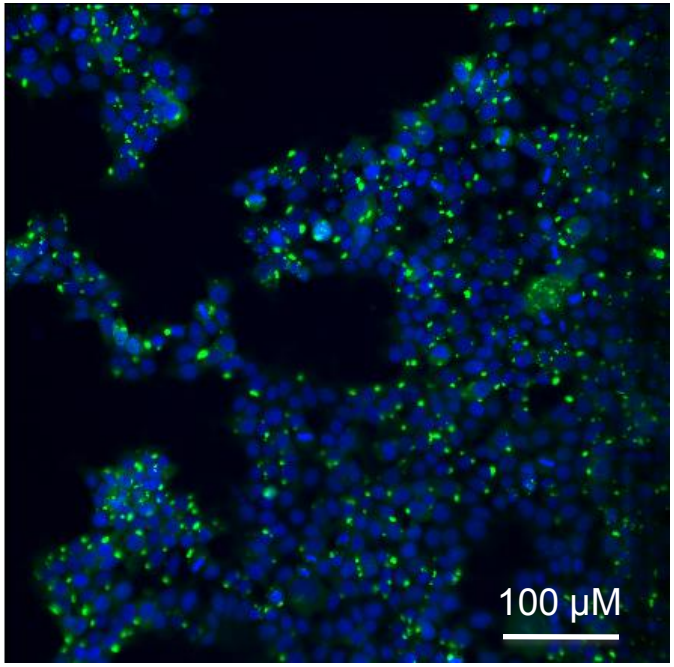
HEK-RD(LM)-YFP clone 1 Infection

2000 HEK Clone 1 cells/well in 384 well plate
Plates analyzed 3 days after exposure to lysate or vehicle

Vehicle



2 µg/well Clone 9 lysate



Hoechst nuclear stain – used at sub-cytotoxic concentration

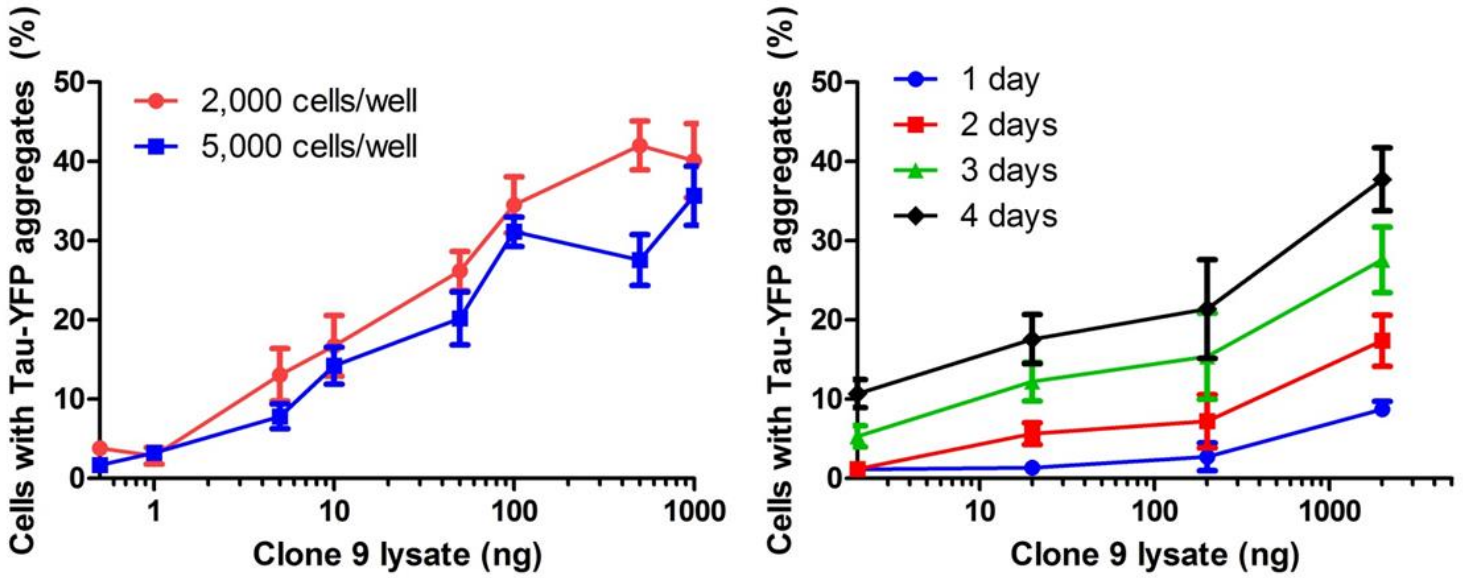
HCA enables the acquisition of many types of data simultaneously

- Mean fluorescence (I)
- Mean area of fluorescence (A)
- Integrated total fluorescence (I x A)
- # of aggregates
- Mean size of aggregates
- Cell area
- Nuclear area

<u>For other cell types (e.g. NPCs)</u>

- Axonal vs somatodendritic staining
- Neurite length
- # of neurites
- # of dendritic branches

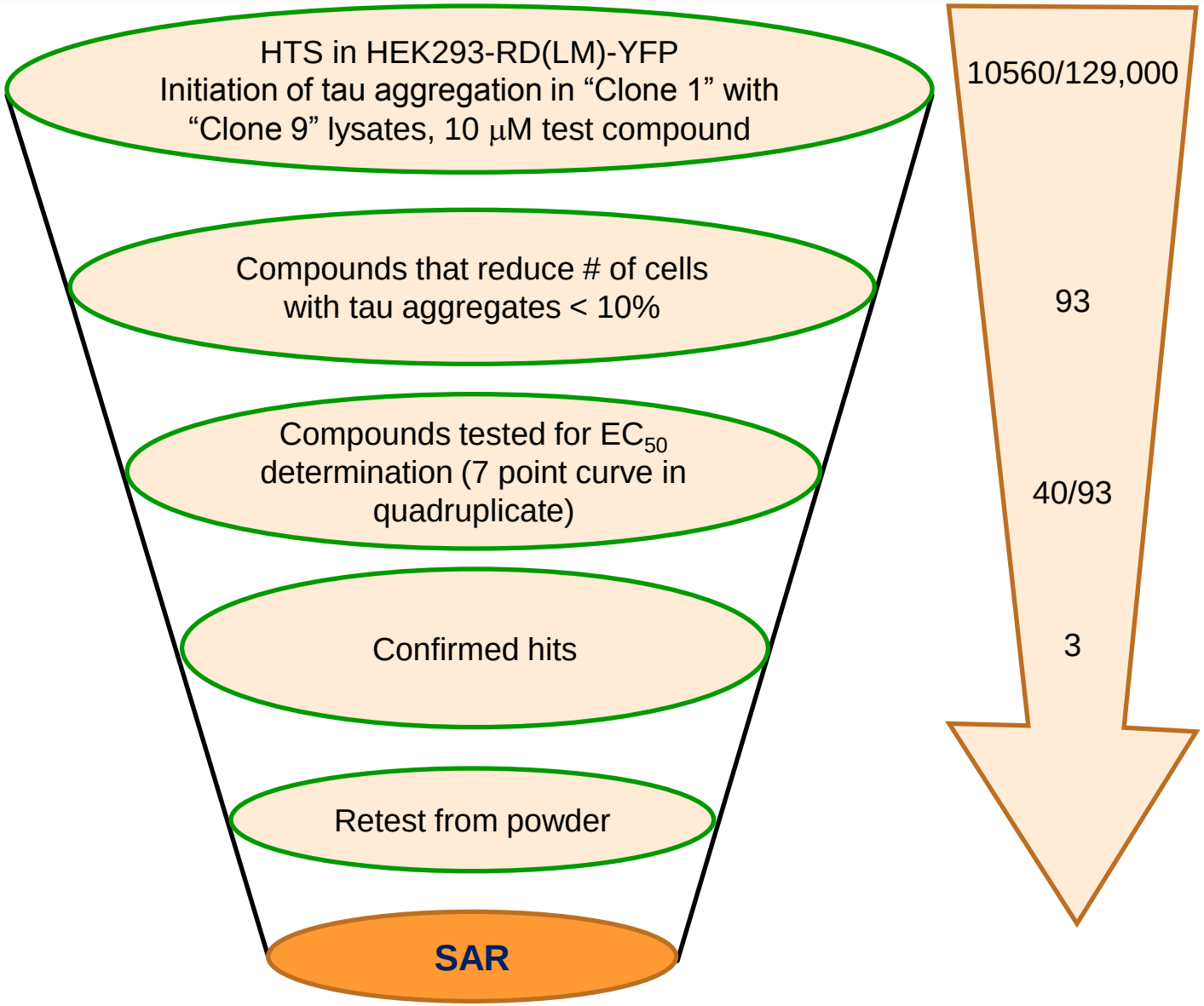
Aggregation Assay Optimization



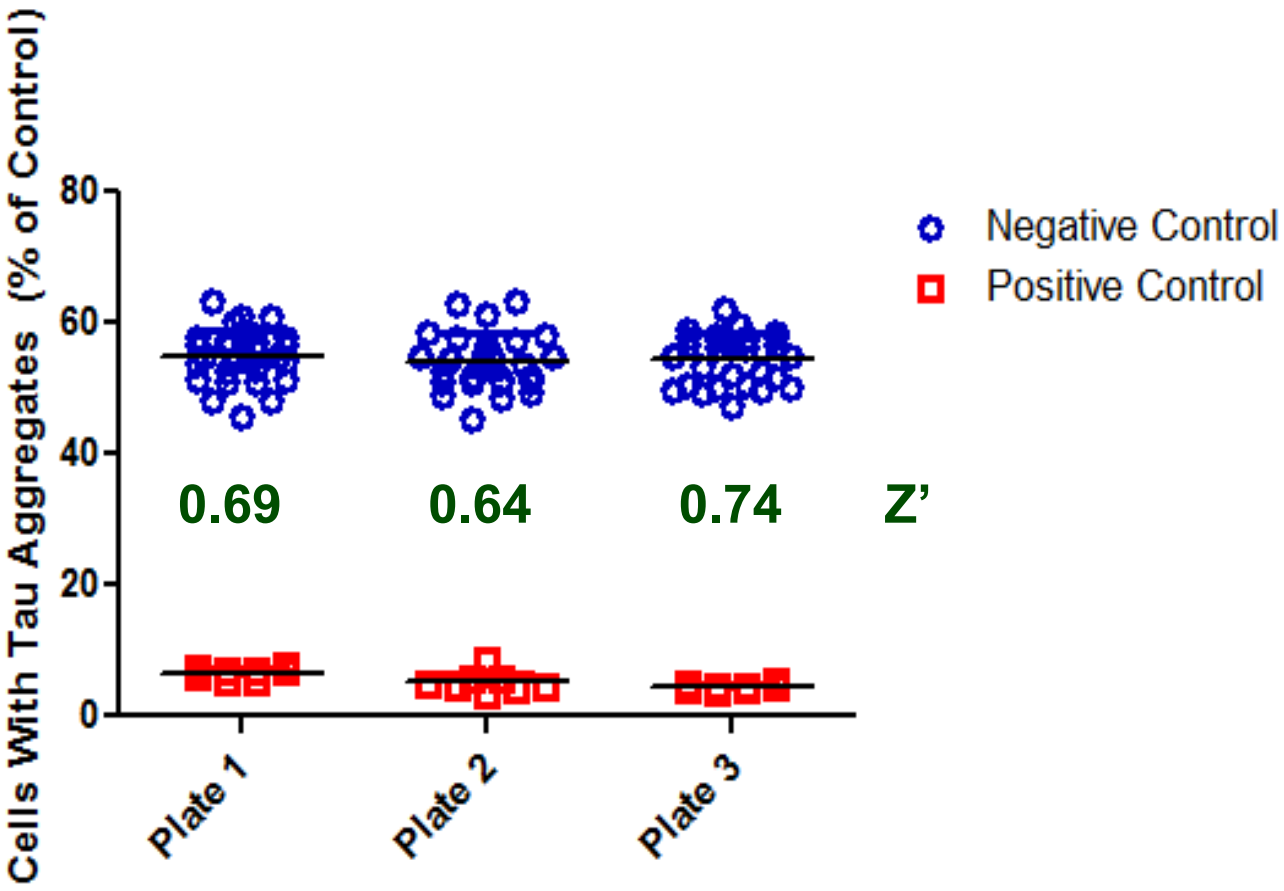
- Magnitude of tau aggregation related to both dose of clone 9 lysate and amount of time post-exposure

- Conditions for HTS:
  - 0.02 and 2 µg/well of clone 9 lysate
  - Plates imaged 2 and 3 days after exposure to lysate

Aggregation Assay Optimization



Aggregation Assay Optimization

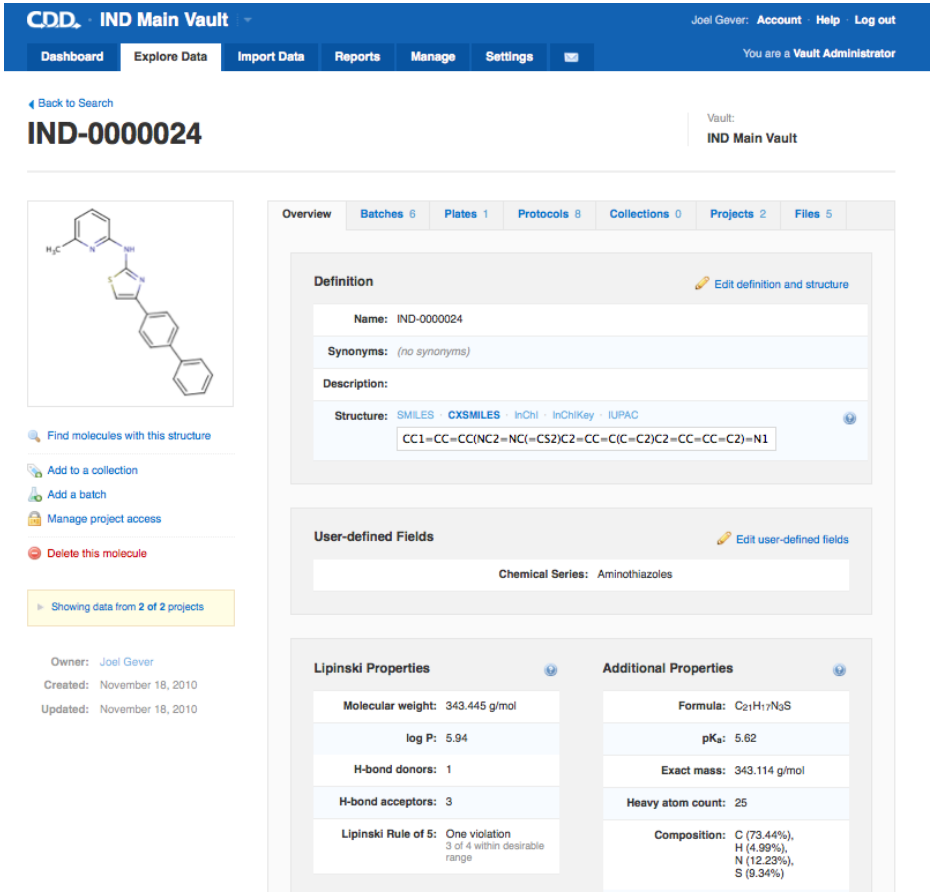


Data storage at IND: finding the right blend of functionality and affordability

CDD is:

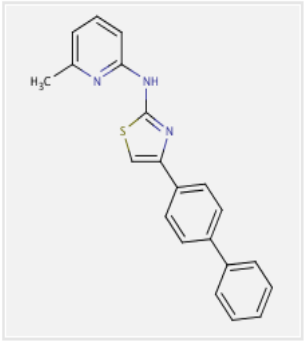
- affordable relational database
- web-based (accessible anywhere)
- secure
- simple
- requires minimal vendor support
- BUT limited customization

20 – 100 TB per year of image data will probably require NAS server or something similar



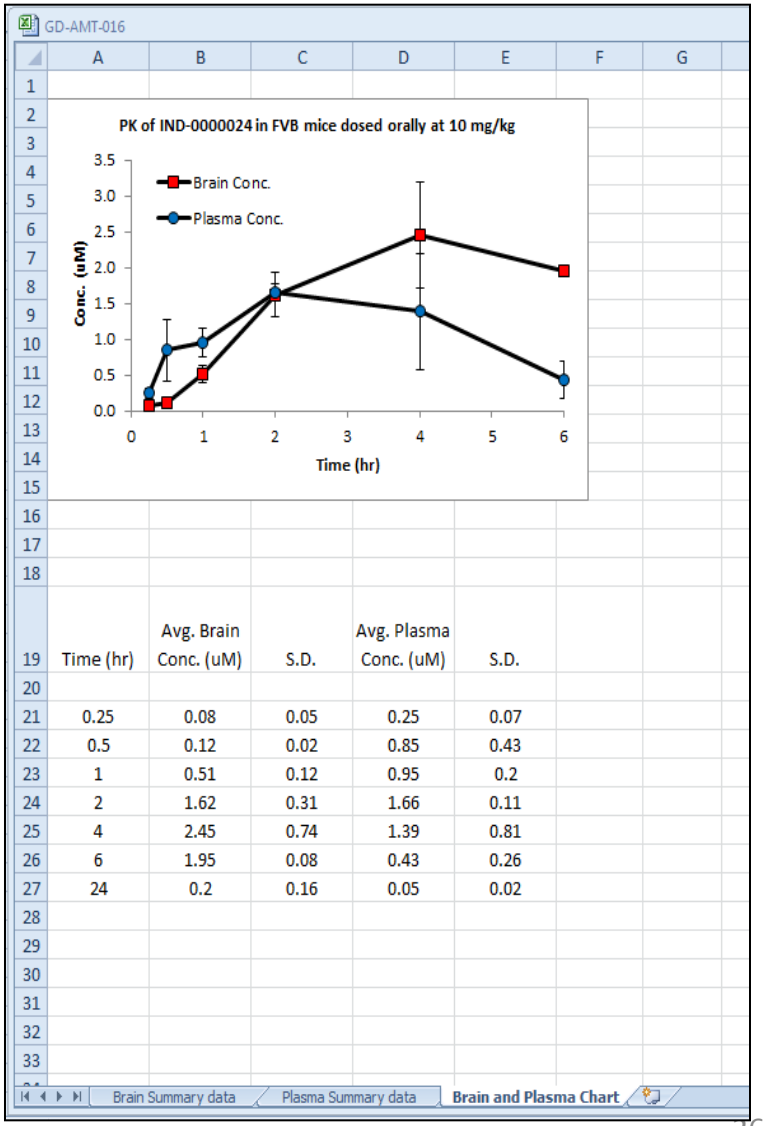
PK data now being stored in CDD

- Study data extracted from Excel spreadsheet using Python script

Select... all · none	Molecule	Batch Fields Name	in vivo PK data Study ID	Species	Animal type	Route	Dosing Method	Dose(s) (mg/kg)	Formulation	Run Date	Study Data File
✓	flag outliers  IND-0000024 IND Main Vault	AA-001	GD-AMT-016	Mouse	FVB	po	gavage	10		10/19/2010	GD-AMT-016.xlsx
			SR-PK-0023	Mouse	FVB	iv	i.v.	1	10% DMSO in 1:1 PEG400/H2O	2/3/2011	SR-PK-0023.xlsx
			SR-PK-0134	Mouse	FVB	iv	i.v.	1	10% DMSO, 20% Ethanol in 1:1 PEG400/H2O	4/7/2011	SR-PK-0134.xlsx
			SR-PK-0026_0027_0028	Mouse	Female FVB mice	po	feeding	75, 125, 210	100% PEG400, diluted 1/800 in chocolate diet	6/2/2011	SR-PK-0026_0027_0028.xlsx
			GD-AMT-005_006_007_008_014	Mouse	Female FVB mice	po	feeding	25, 75, 125, 210	100% PEG400, diluted 1/80 in chocolate diet	July 29-30, 2010	GD-AMT-005_006_007_008_014.xlsx
			SR-PK-0002	Mouse	FVB	po	feeding	75	100% PEG400, diluted 1/80 in chocolate diet	10/28/2010	SR-PK-0002_0003_0004_0005.xlsx
			SR-PK-0026	Mouse	FVB	po	feeding	75, 125, 210	100% PEG400, diluted 1/800 in chocolate diet	12/9/2010	SR-PK-0026_0027_...y3_14_32_90.xlsx
			SR-PK-0061	Mouse	FVB	po	feeding	75, 75, 75, 75	100% PEG400, diluted 1/80 in chocolate diet	1/6/2011	SR-PK-0061_0062_0063_0064.xlsx
			SR-PK-0074	Mouse	FVB	po	feeding	75, 75, 75, 75	100% PEG400, diluted 1/80 in chocolate diet	1/20/2011	SR-PK-0074-0081.xlsx

Link to raw data is helpful

- Raw data and graphs are easily retrieved



Time (hr)	Avg. Brain Conc. (uM)	S.D.	Avg. Plasma Conc. (uM)	S.D.
0.25	0.08	0.05	0.25	0.07
0.5	0.12	0.02	0.85	0.43
1	0.51	0.12	0.95	0.2
2	1.62	0.31	1.66	0.11
4	2.45	0.74	1.39	0.81
6	1.95	0.08	0.43	0.26
24	0.2	0.16	0.05	0.02

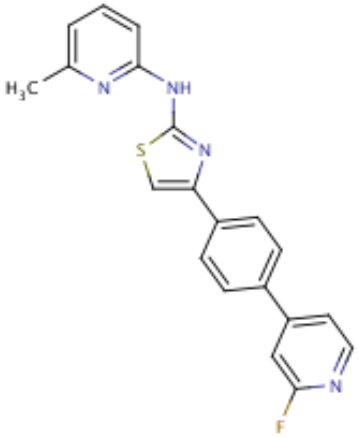
Conclusions

- Screening format has transitioned from relatively primitive ELISAs to sophisticated, data-rich high content analysis
- Automation has been employed to maximize advantages of high content analysis
- TBs of image data necessitates more sophisticated data storage

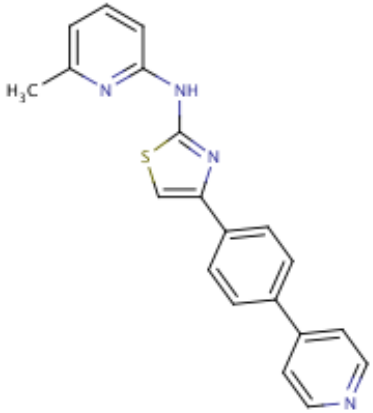
Back up slides

Several promising leads from PrP^{Sc} reduction HTS

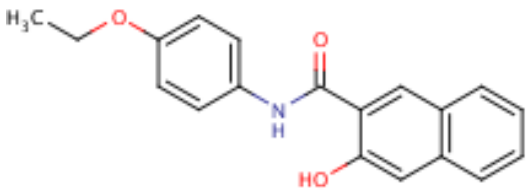
- 66,496 compounds screened in dividing ScN2a cells using an ELISA
  - 14 structural types were investigated to varying degrees
  - Good variety of structural types found, with very good potency for lead compounds



IND-0114335 aka **"AMT1"**
(aminothiazole)

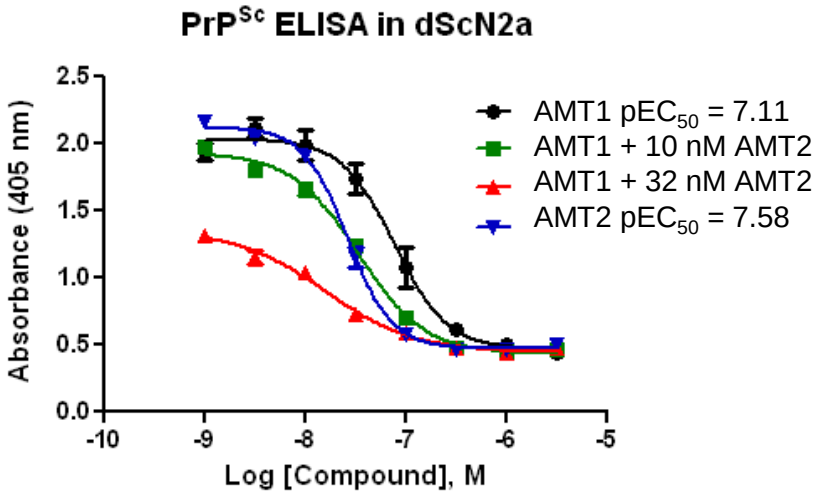


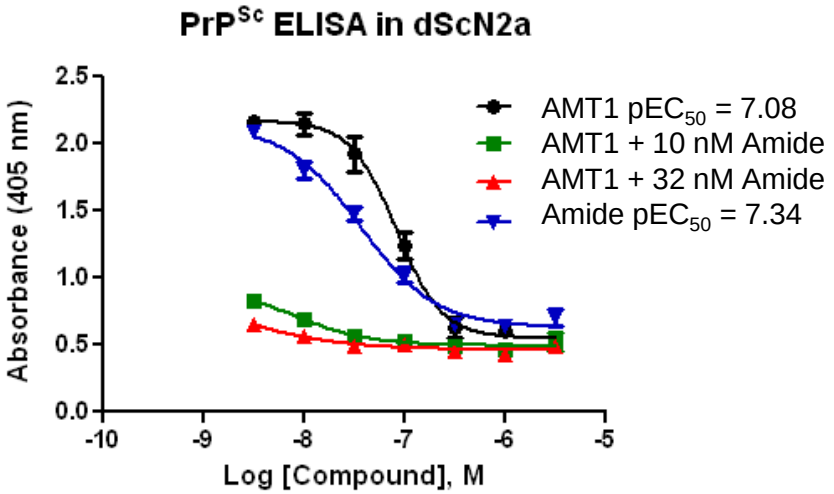
IND-0114337 aka **"AMT2"**
(aminothiazole)

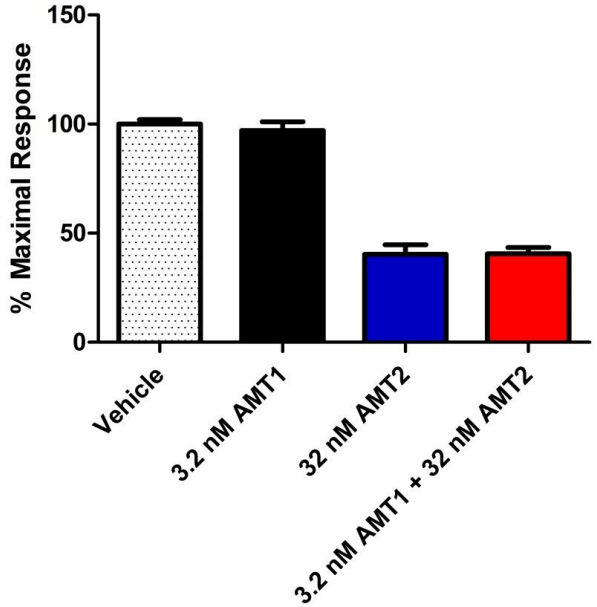


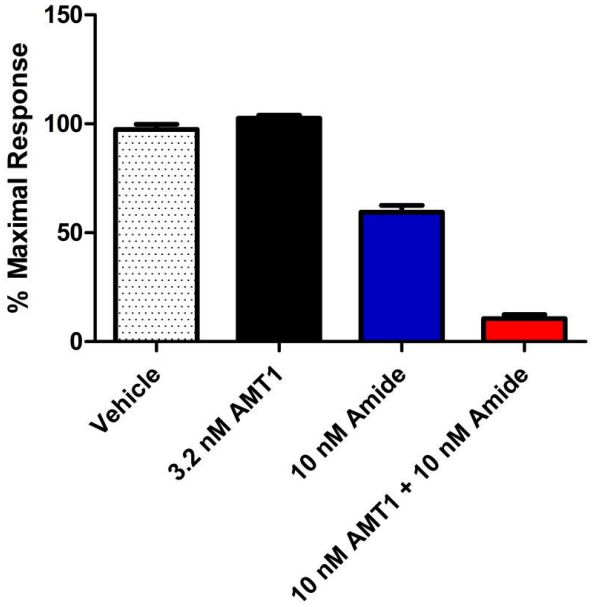
IND-0031751 aka **"Amide"**
(amide)

Pharmacological combination hints at similarity/dissimilarity of mechanism

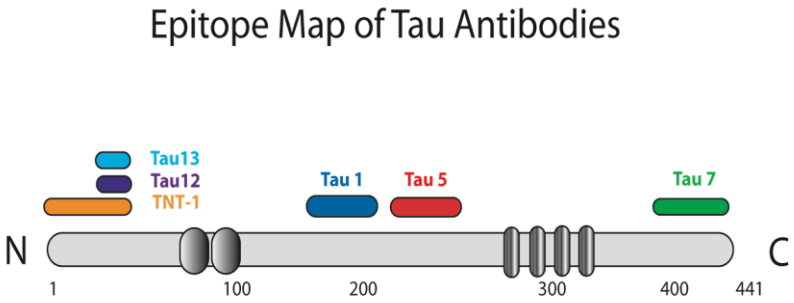




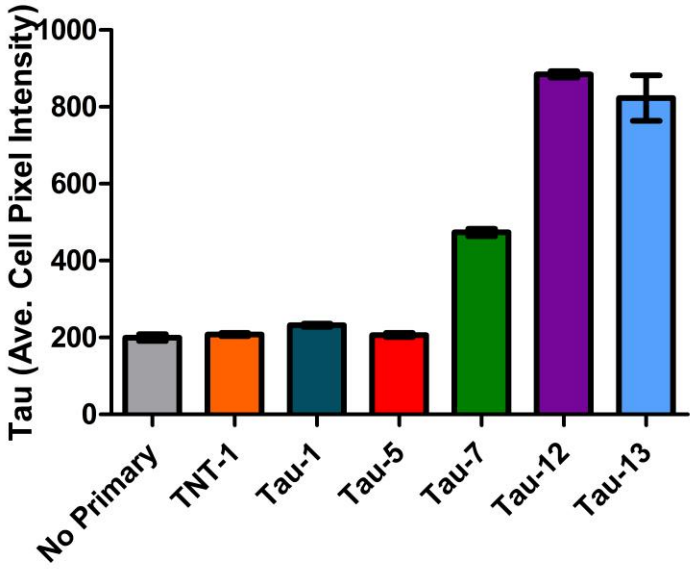




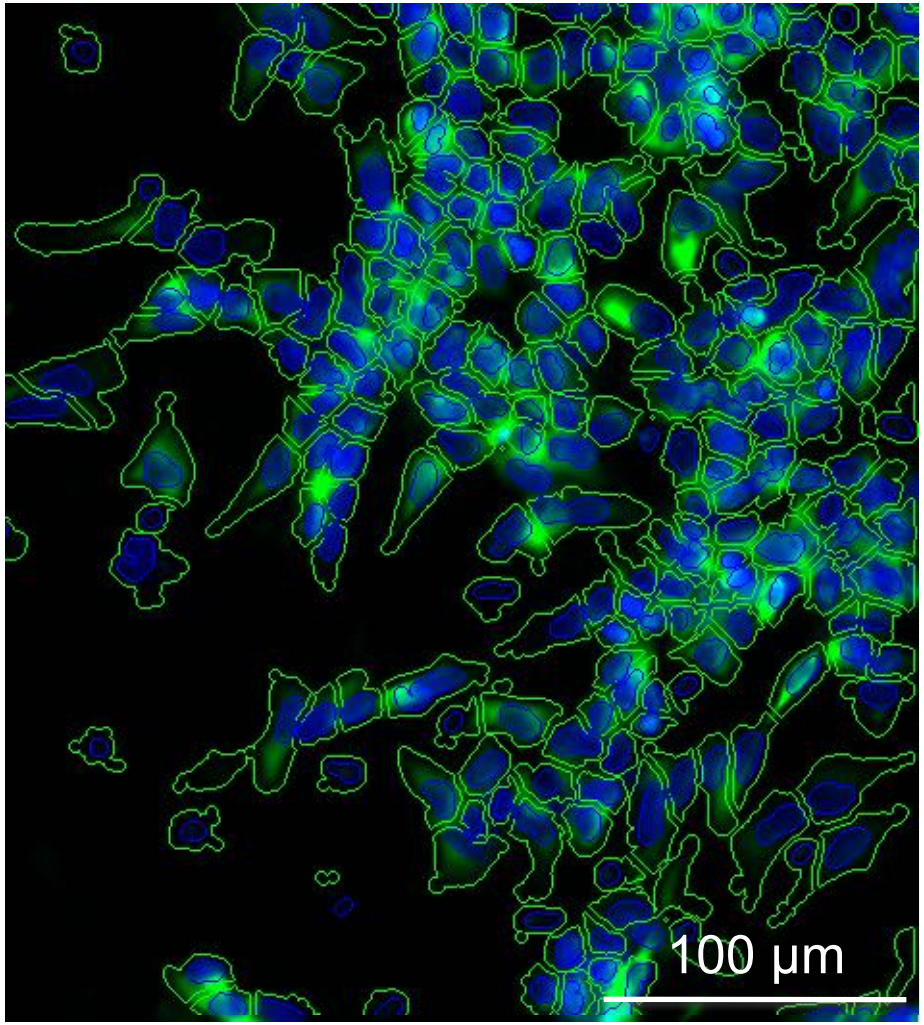
Measuring tau reduction by HCA



Tau-12 & Tau-13 gave strongest signal



Tau antibodies kindly provided by Skip Binder, Northwestern University

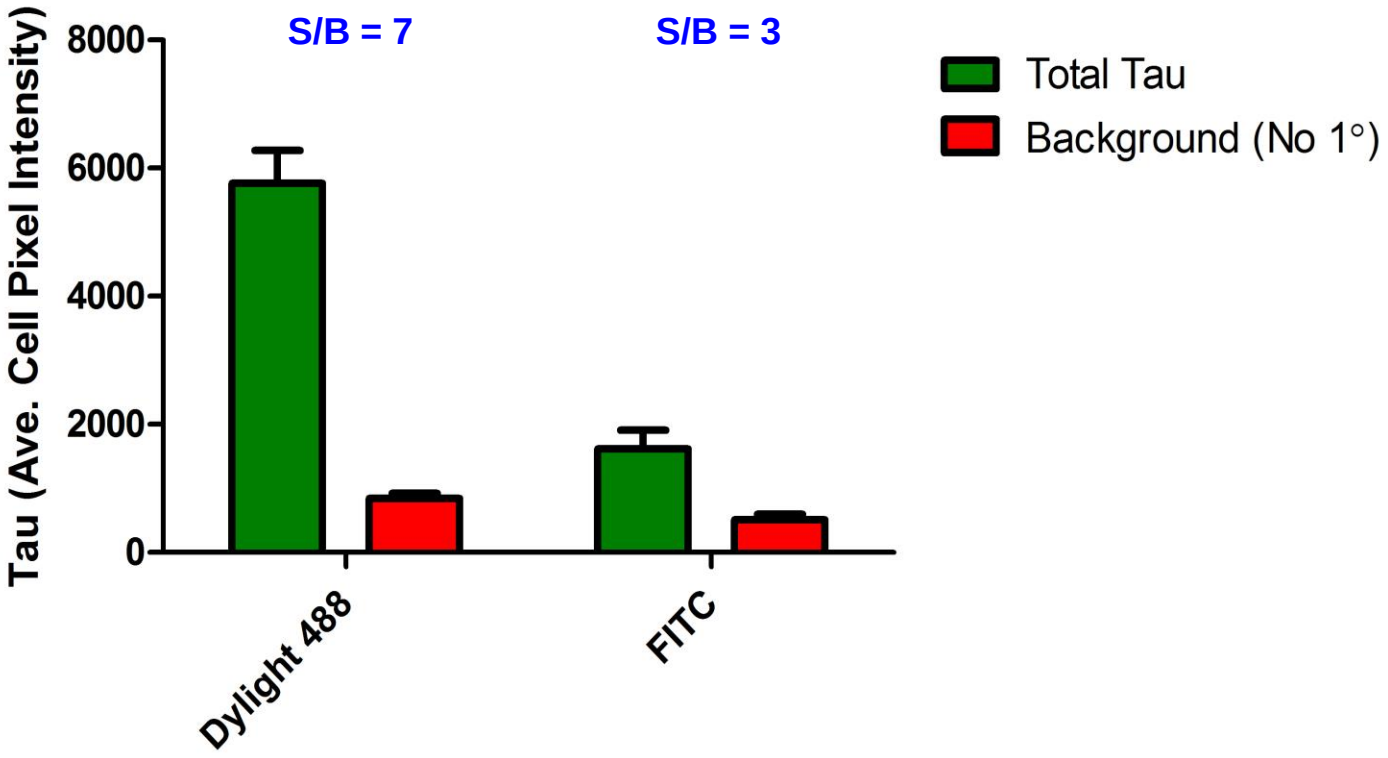


SH-SY5Y human neuroblastoma cells:

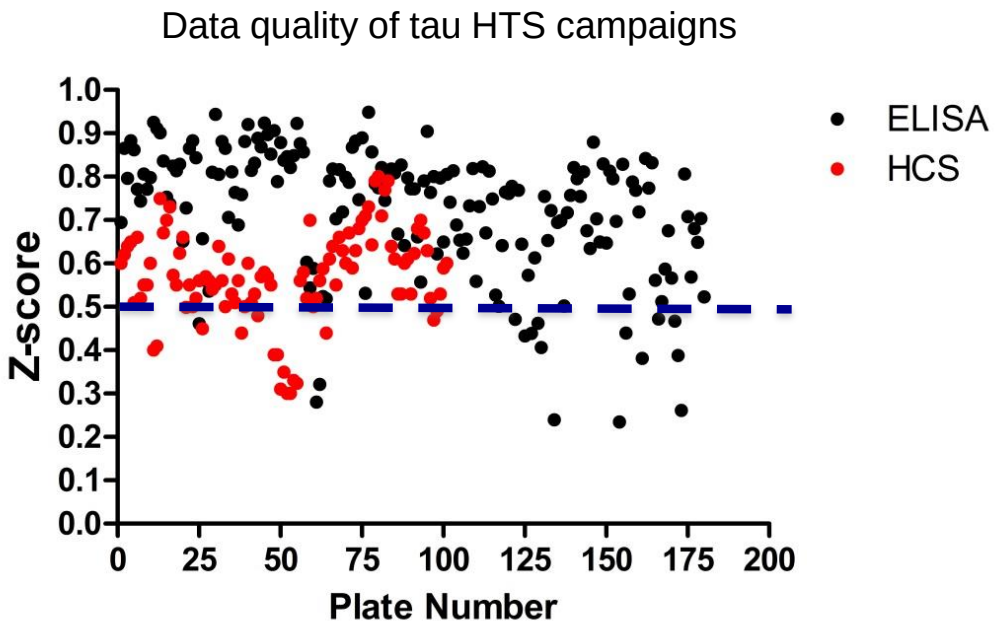
- **Nucleus** (DAPI)
- **Tau** (1˚ : Tau-13; 2˚ GaM-Alexafluor488)

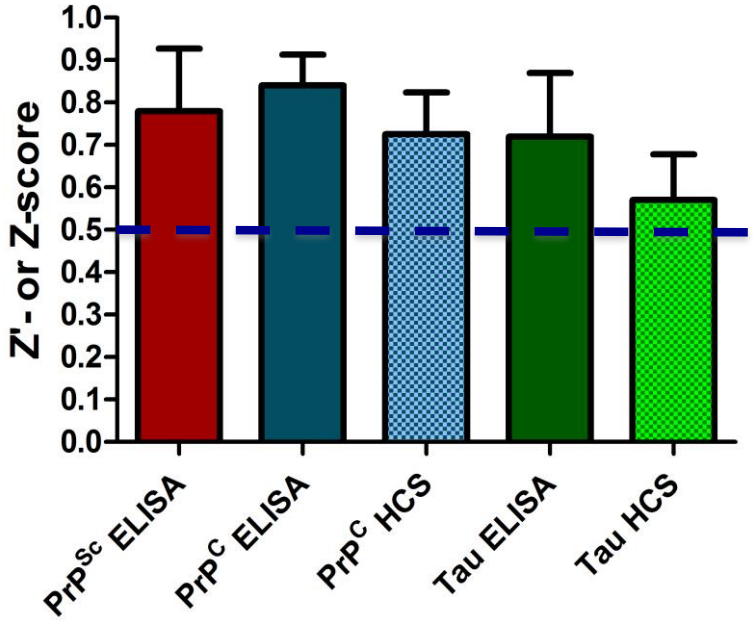
Tau HCS update

- Triplicates from 384 well plate, SH-SY5Y cells
- Primary = Tau13
- Secondary = goat anti-mouse-FITC or goat anti-mouse-Dylight 488



Data quality of Tau HCS is comparable to previous screens

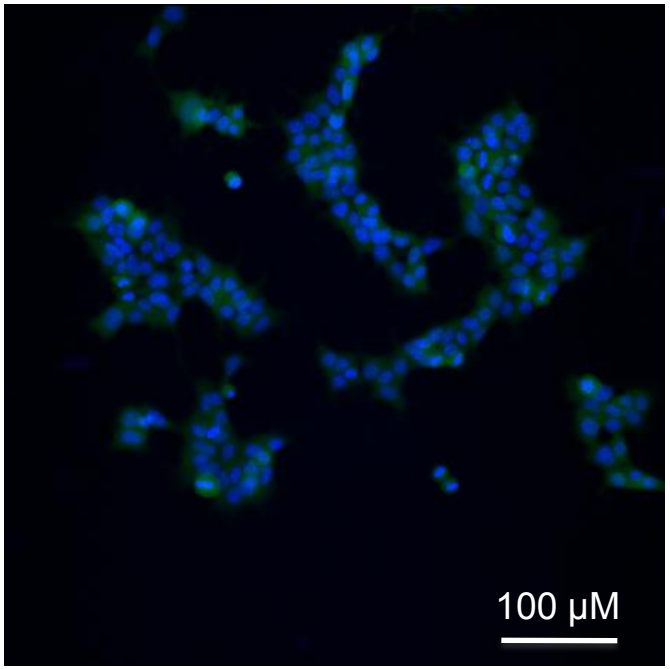




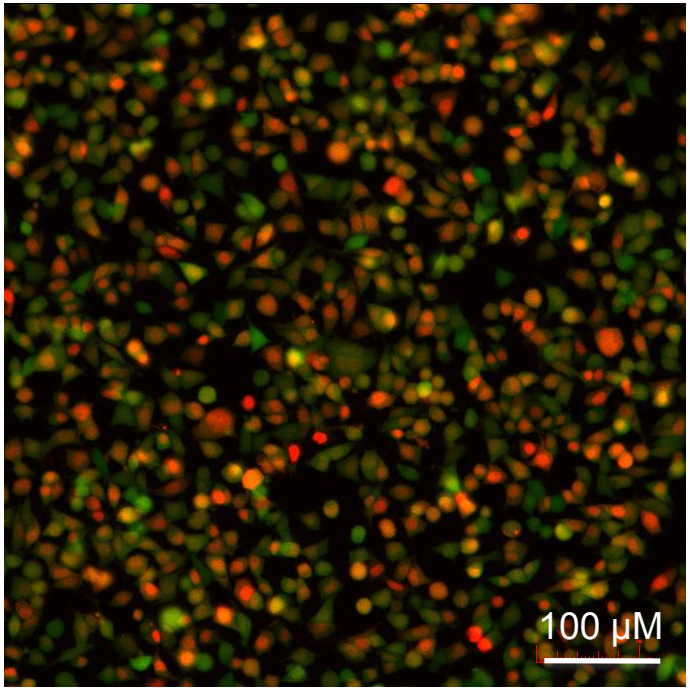
- Tau reduction by HCA
  - 43,504 molecules tested in SH-SY5Y human neuroblastoma cells: single well, 10 µM
  - 652 hits (≥ 40% reduction of tau, < 50% reduction # of cells)

HEK-RD(LM)-YFP-mCherryNLS

Nuclei stained with Hoechst



Nuclei expressing mCherry-NLS



mCherry with a nuclear localization sequence enables automated nuclear localization

HEK-RD(LM)-YFP cells expressing mCherry-NLS kindly provided by Marc Diamond, Washington University