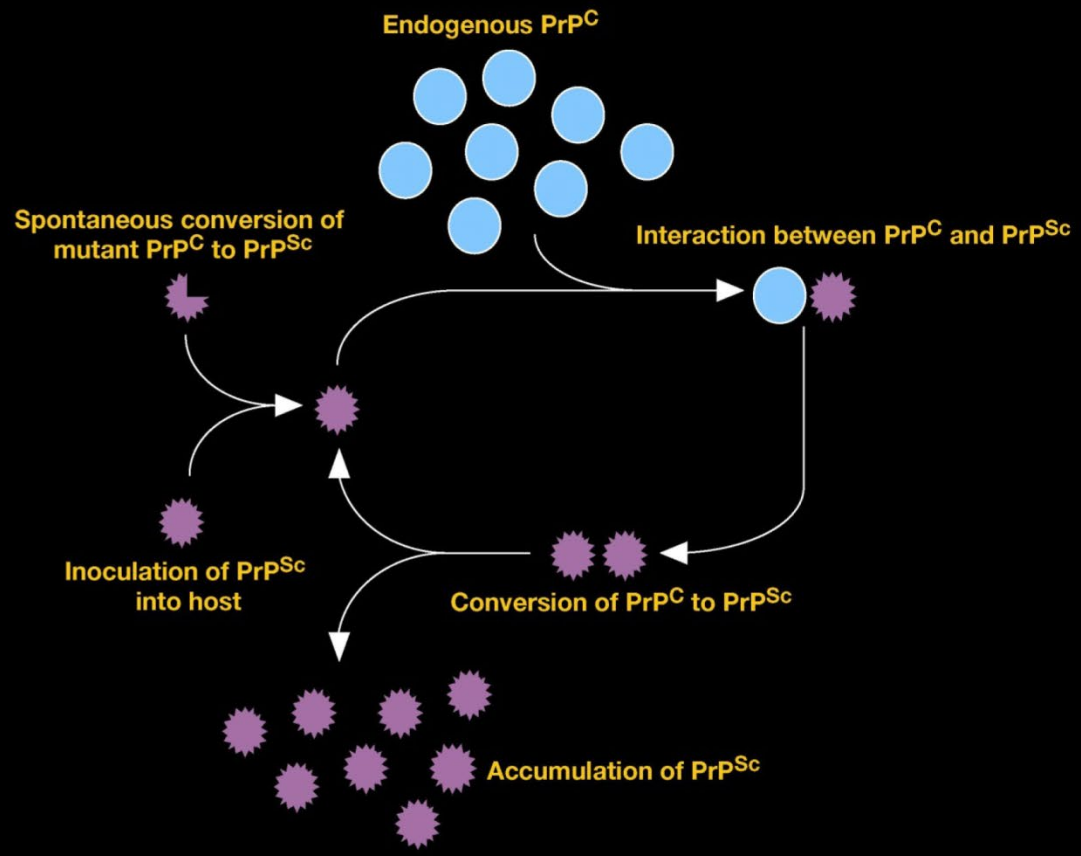
Development of a quantitative, real-time Protein-Misfolding by Cyclic Amplification (PMCA) reaction.

Dr Graham Jackson

MRC Prion Unit at UCL

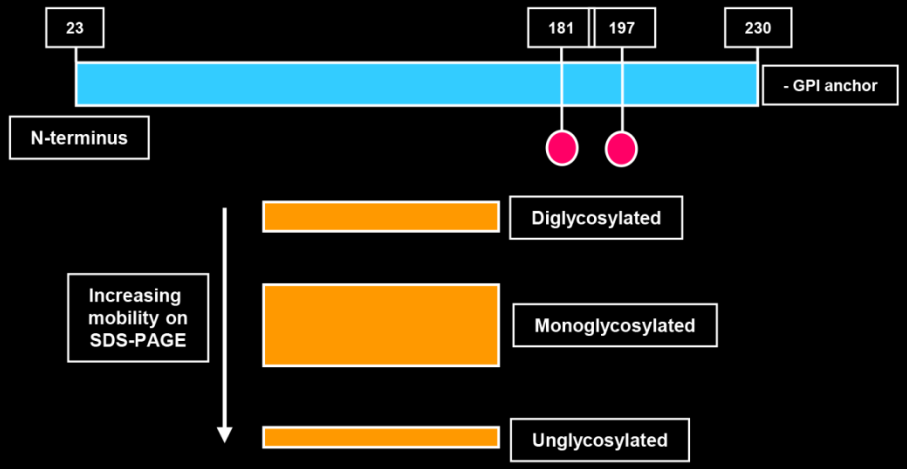
13th July 2019

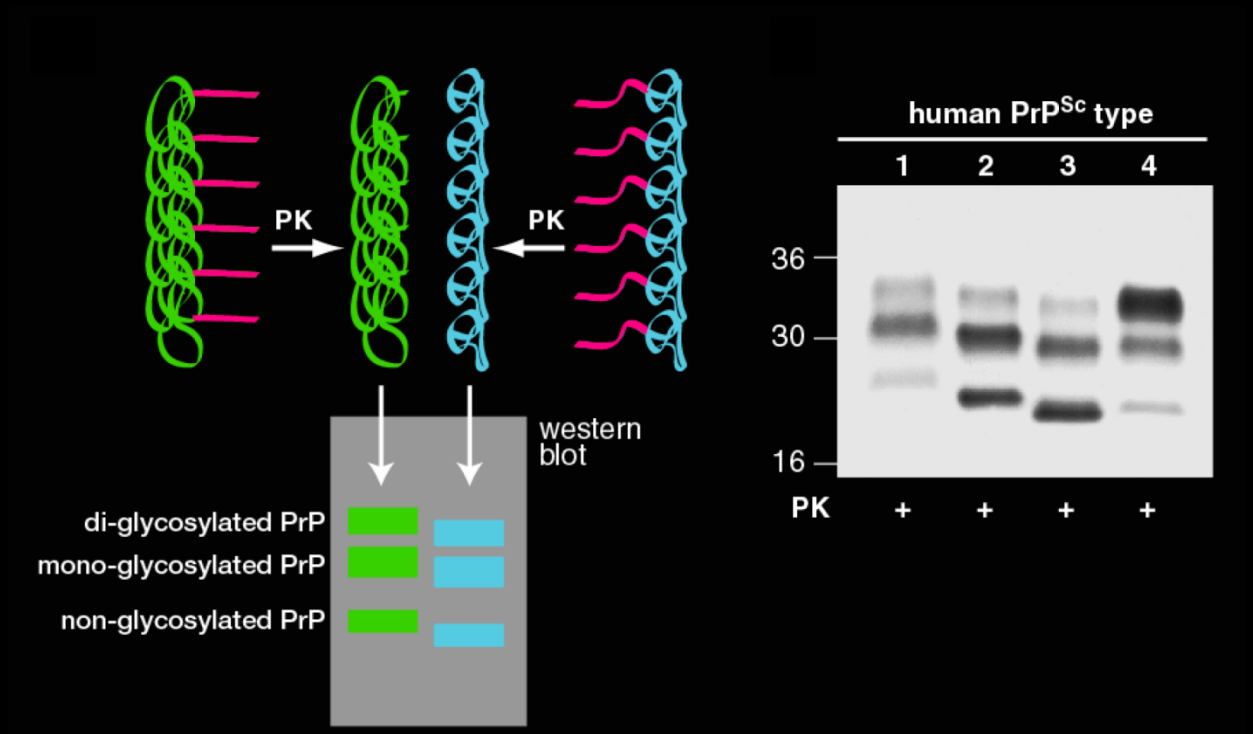
Protein-only model of prion propagation



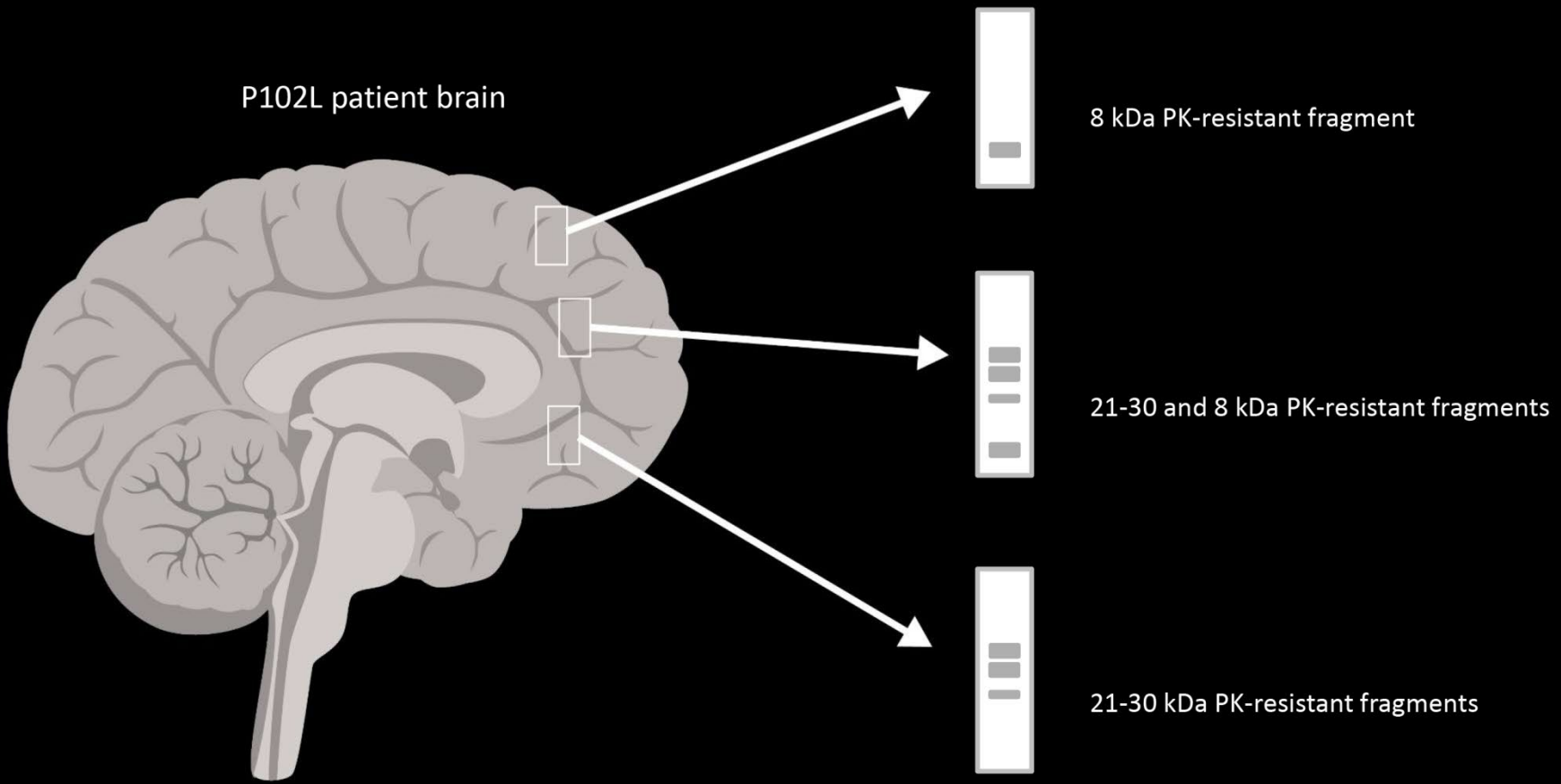
PrP^{Sc} replicates with high fidelity by recruitment of endogenous PrP^{C}

Human prion protein post-translational modifications





Prions and amyloid are distinct and commonly co-exist



PrP amyloid is transmissible but not pathogenic

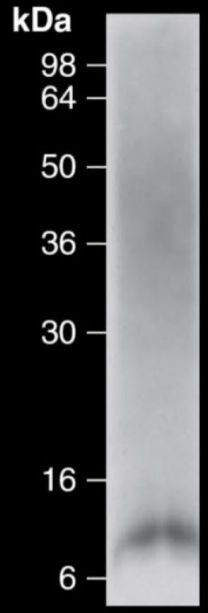
P102L patient brain sample

Tg(HuPrP$^{102L\ 129M+/+}$ *Prnp*$^{o/o}$)-27 mice

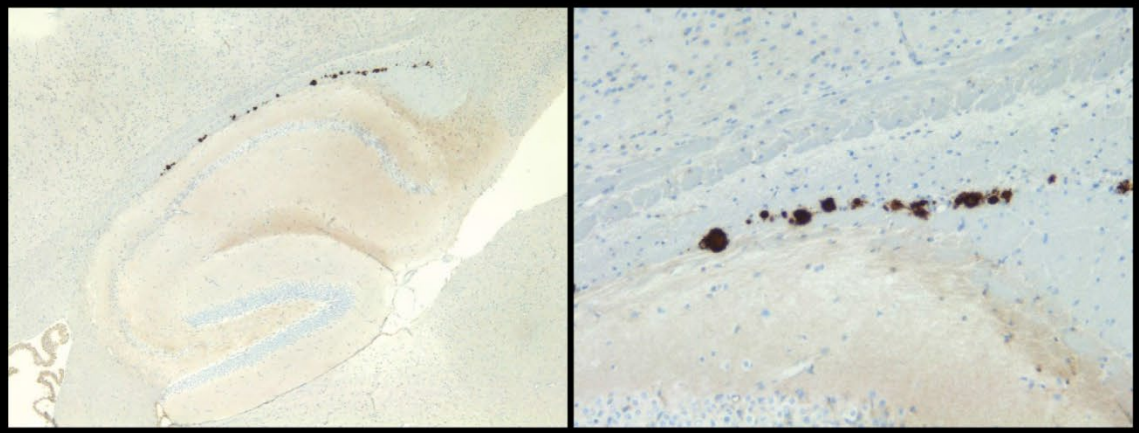
0/19 clinical disease > 285-604 days

19/19 PrP 102L plaques

P102L patient brain sample



Transmission to Tg(HuPrP$^{102L\ 129M+/+}$ *Prnp*$^{o/o}$)-27 mice



Diagnostic markers of prion disease

- Tissue biopsy (western blot detection of PrP^{Sc}) *
- MRI
- Surrogate markers (14-3-3, Nf-L, Tau, etc)
- DDA (abnormal PrP)
- Amyloid seeding (QuIC)
- PMCA * (Prions)
- Cell-culture assay * (Prions)

* Can differentiate between prion strains

Elongated Oligomers Assemble into Mammalian PrP Amyloid Fibrils

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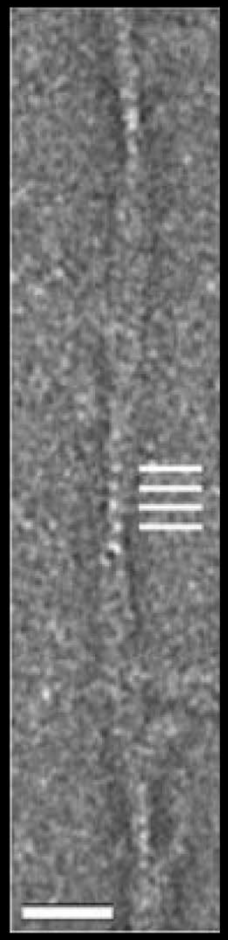
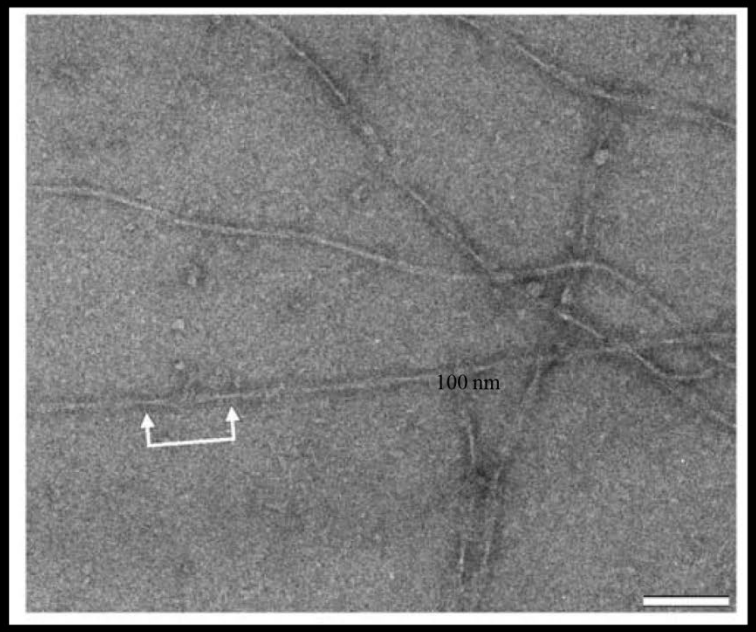
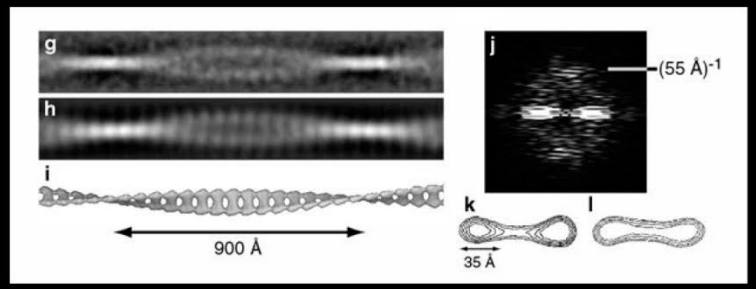
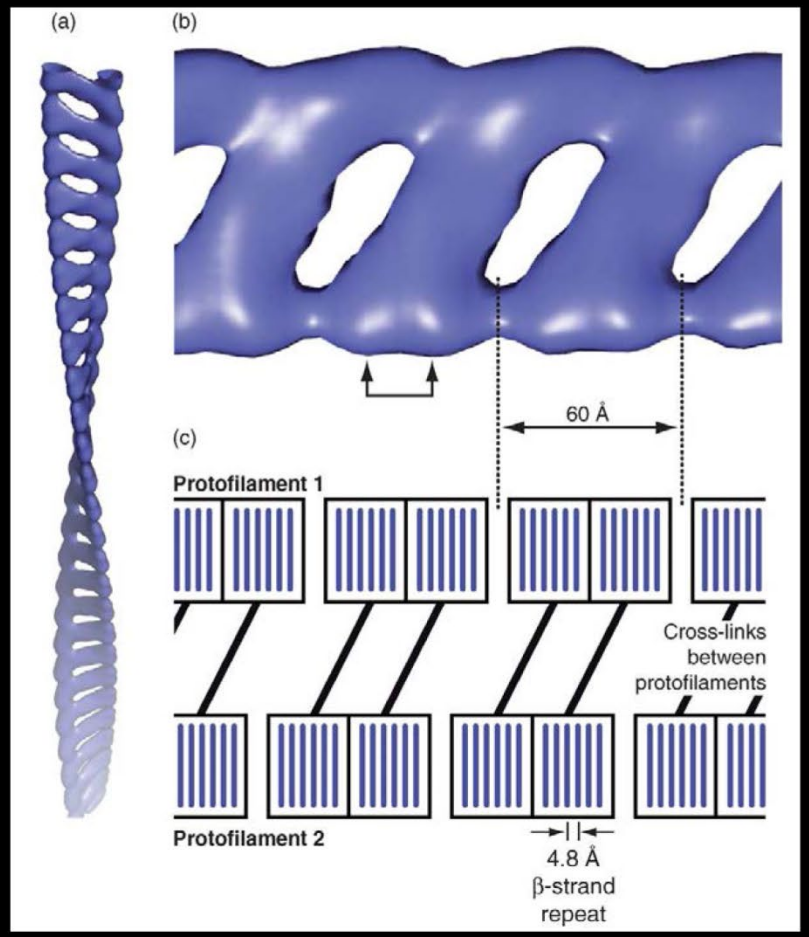
Graham S. Jackson[1], Elena V. Orlova[2], John Collinge[1]
Anthony R. Clarke[1] and Helen R. Saibil[2]*



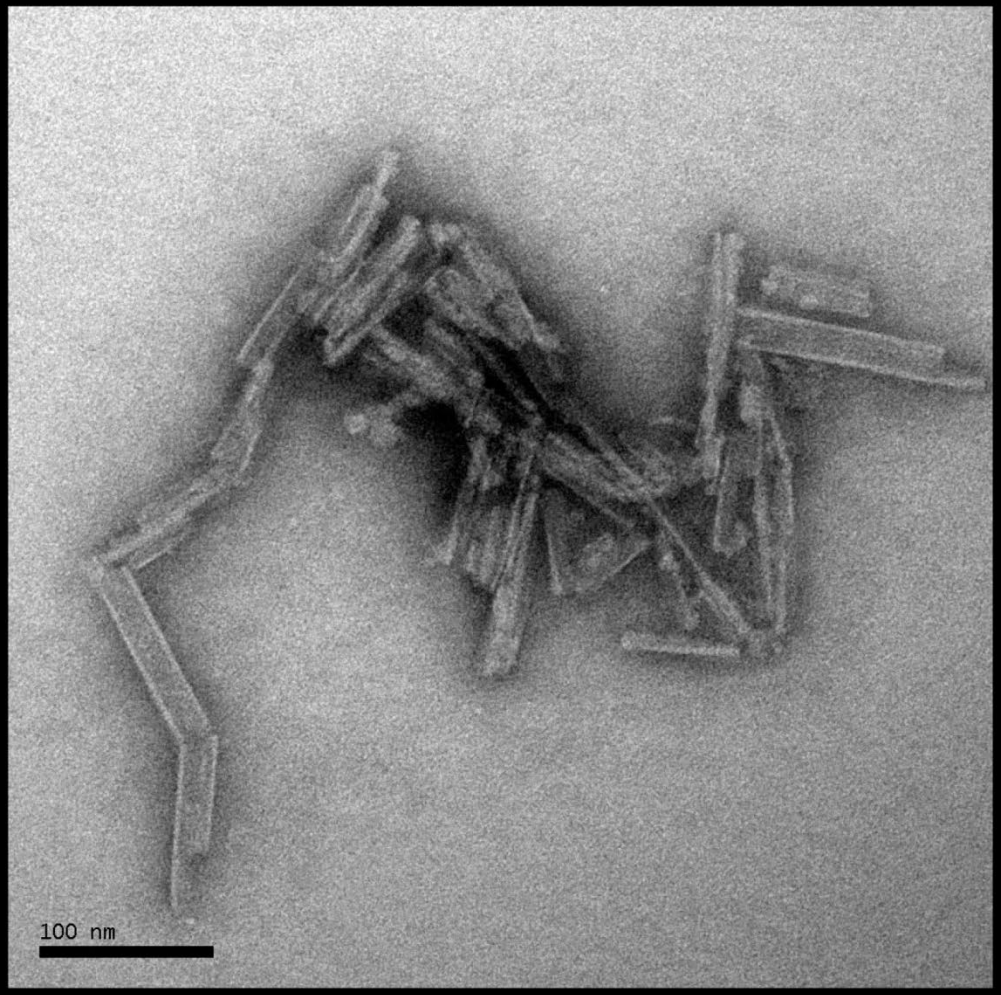


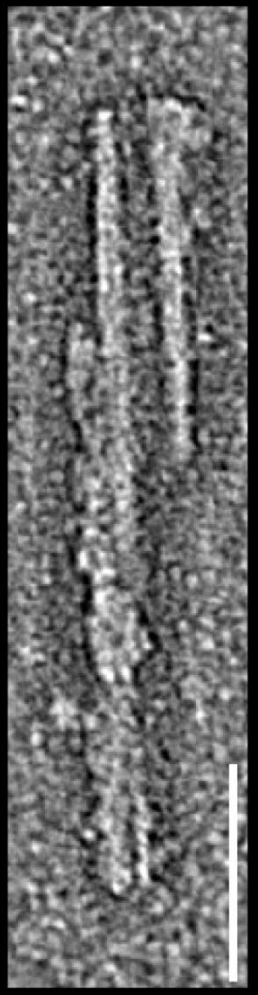




Journal of Molecular Biology 2006

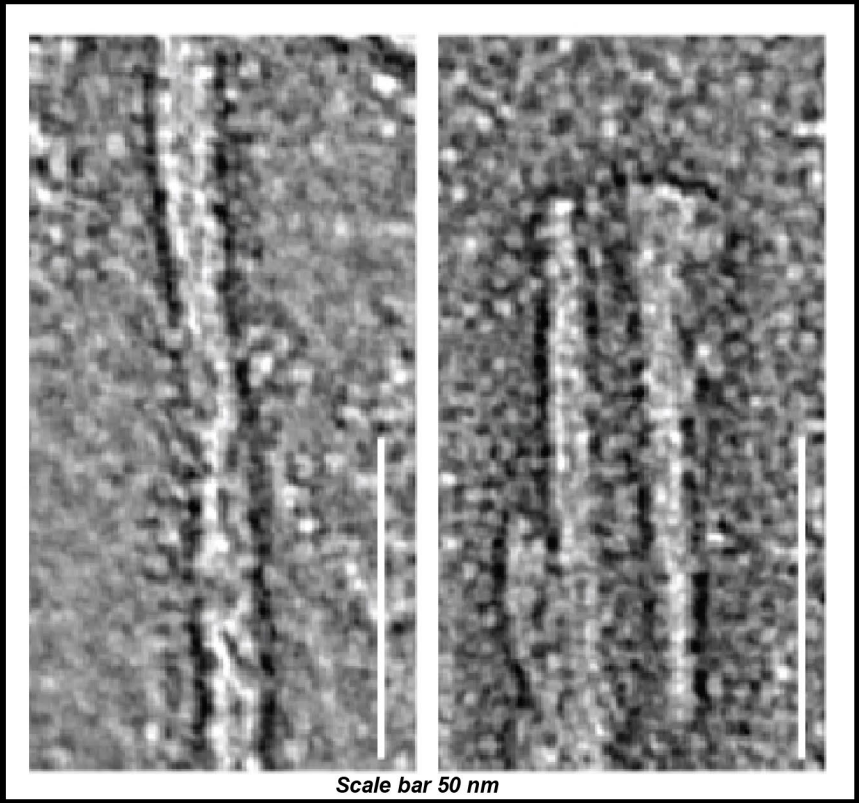
Defining 3D structure of infectious prions: negative stain EM





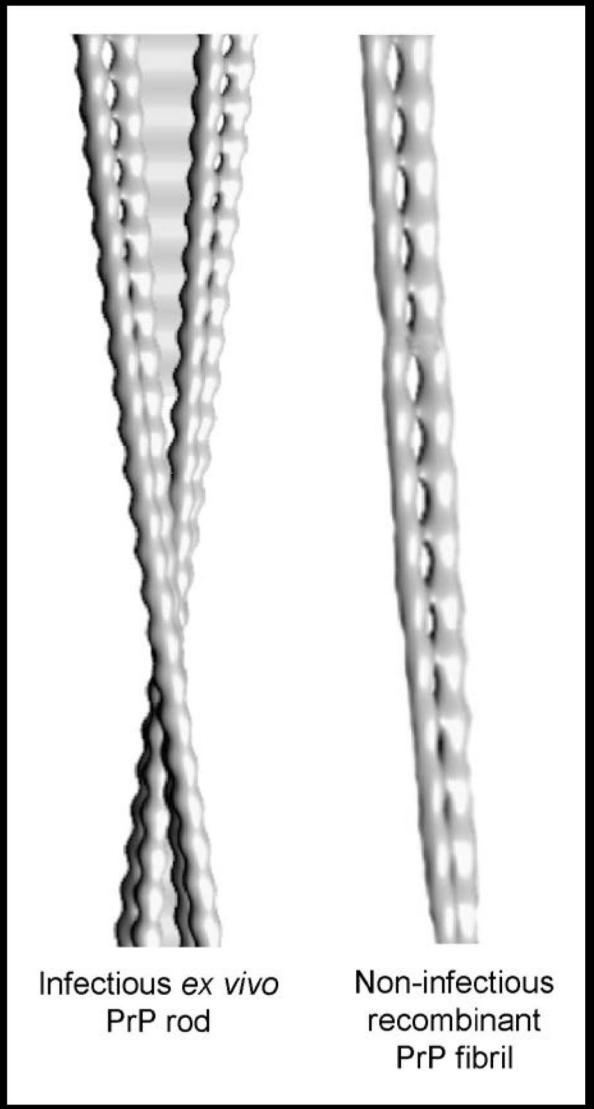
Wenborn et al, Scientific Reports 2015 and Terry et al, Open Biology 2016

Dimensions from dual-tilt electron tomography



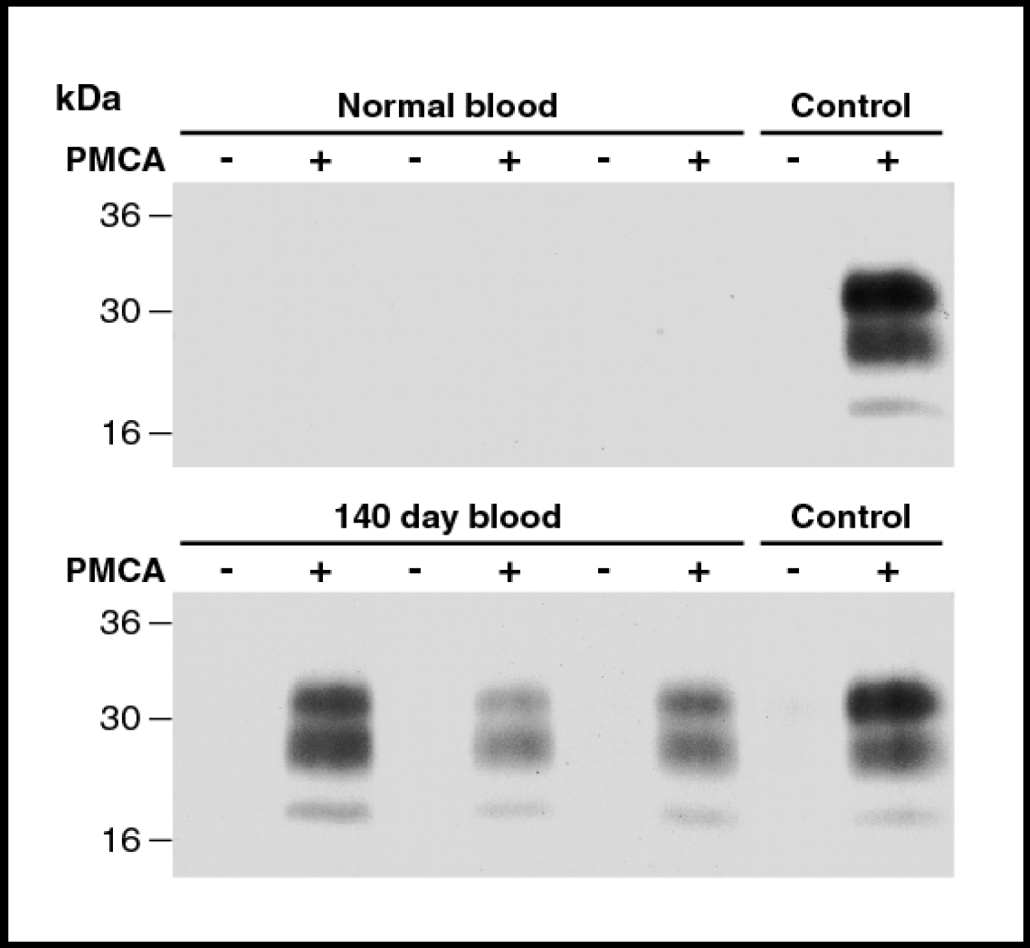
rec PrP fibril
Non-infectious
1 fibre
> 1 µm long
10-12 nm wide
7 nm thick

PrP rod
Infectious
2 fibres
< 0.2 µm long
20-25 nm wide
10-11 nm thick



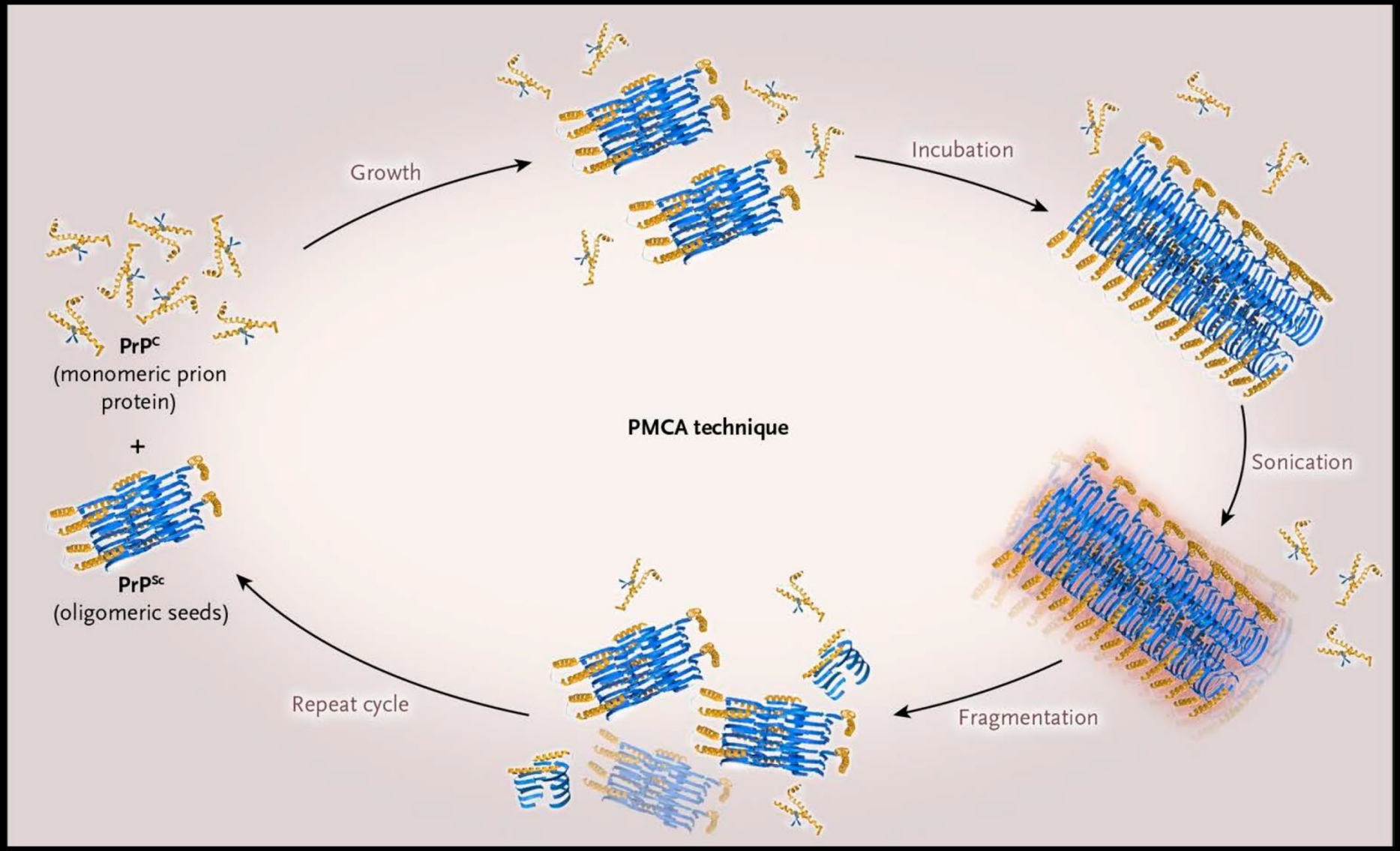
Terry et al, Scientific Reports 2019

Protein Misfolding by Cyclic Amplification (PMCA)



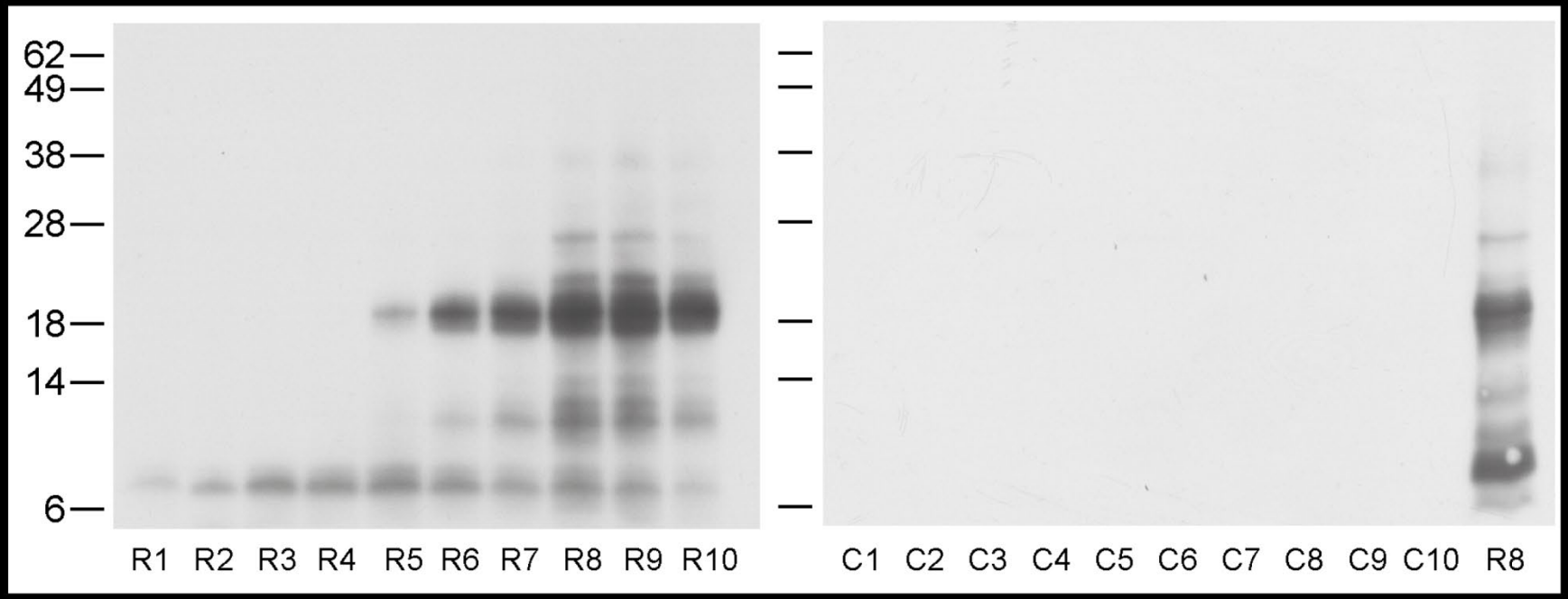
- High sensitivity
- Detects authentic prions
- Time consuming
- Requirement for ex vivo substrate
- Not quantitative
- Substrate difficult to manipulate

Tattum et al, Transfusion 2010



Moda et al, New England Journal of Medicine 2014

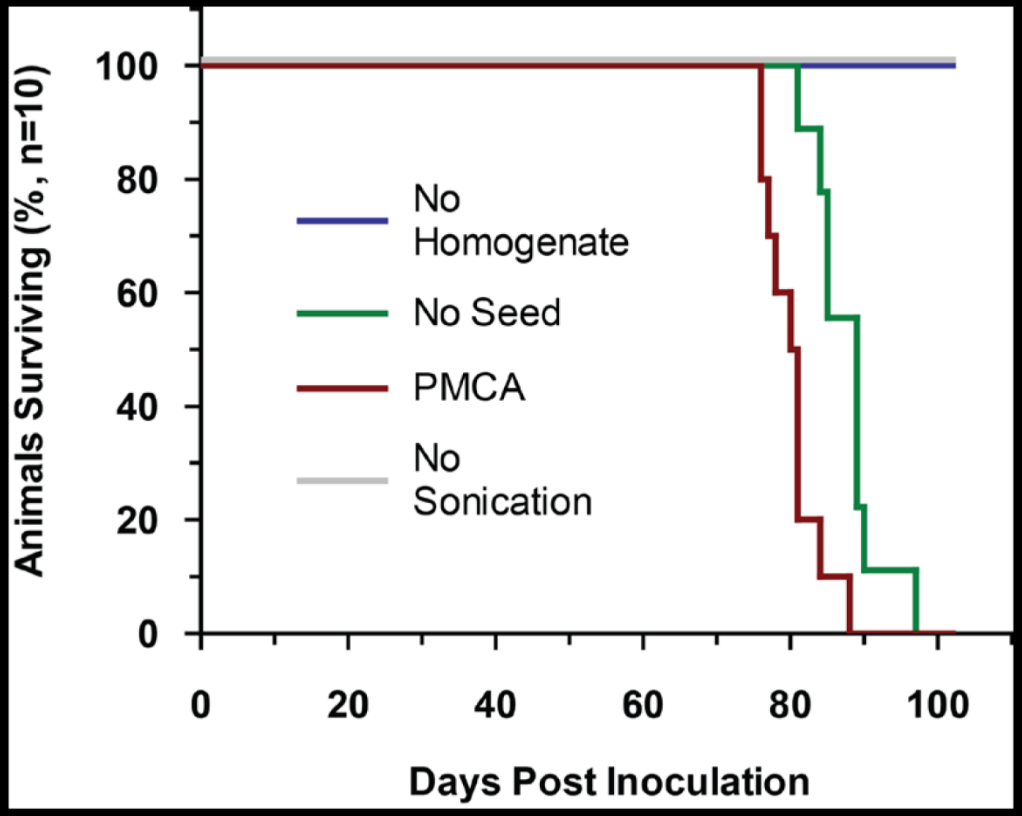
Generation of synthetic prions (SYPRIONS) by PMCA



- High sensitivity
- Detects authentic prions
- Time consuming

- *Requirement for ex vivo substrate*
- **Not quantitative**
- *Substrate easy to manipulate*

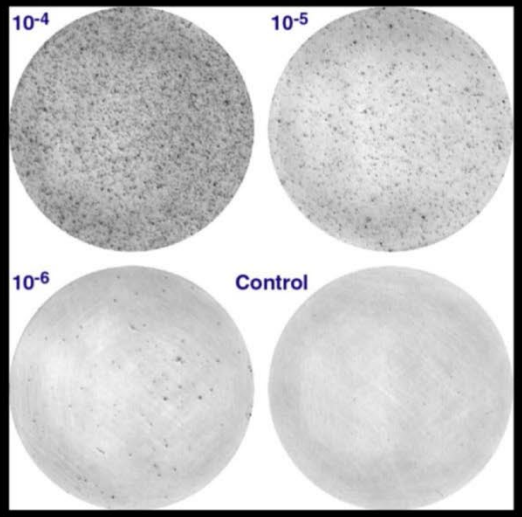
Bioassay confirmed prion infectivity

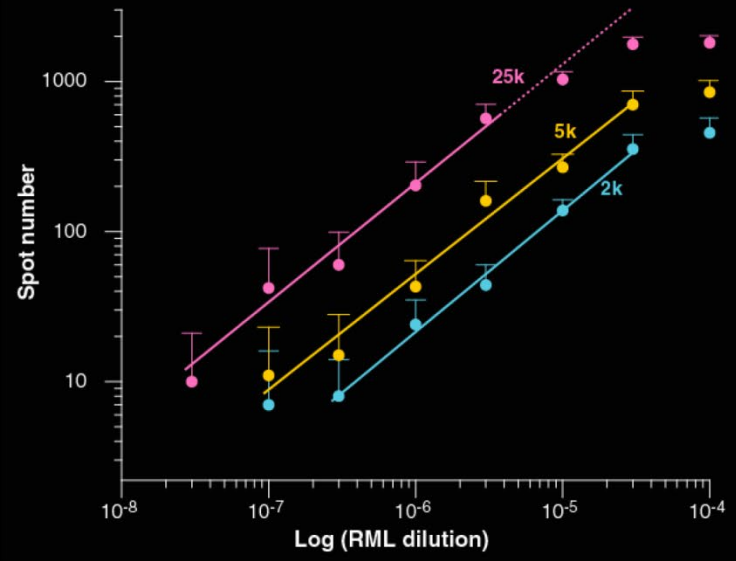


Inoculum (PMCA products diluted 1:1000)	Affected Animals / Total Animals (Tg20)	Mean Incubation Period +/- SD (Days)
PMCA Reaction	10/10	80.2 +/- 3.8
No Seed	9/9	87.7 +/- 4.6
No Sonication	1/9	85
No Homogenate	0/10	NA

The Scrapie Cell Assay (SCA)

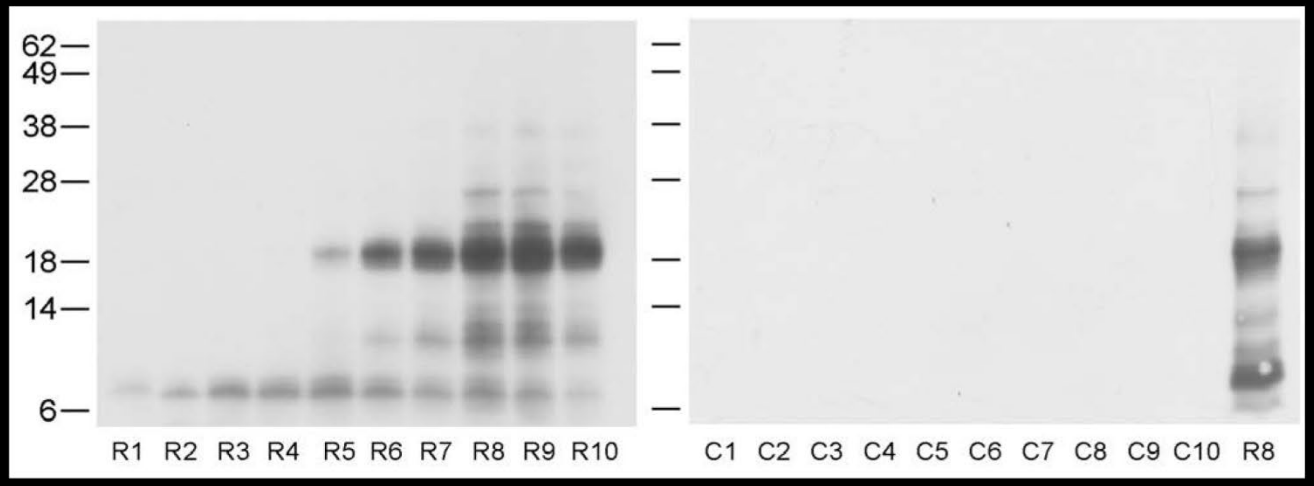
- Cell-culture assay for rodent prions originally based upon a highly susceptible sub-clone of N2a cells (PK1).
- The Scrapie Cell Assay is based on the finding that discrete PrP^{Sc}-positive cells can be detected under the microscope.
- This enables quantification of the prion concentration in an unknown sample which is proportional to the number of infected cells.

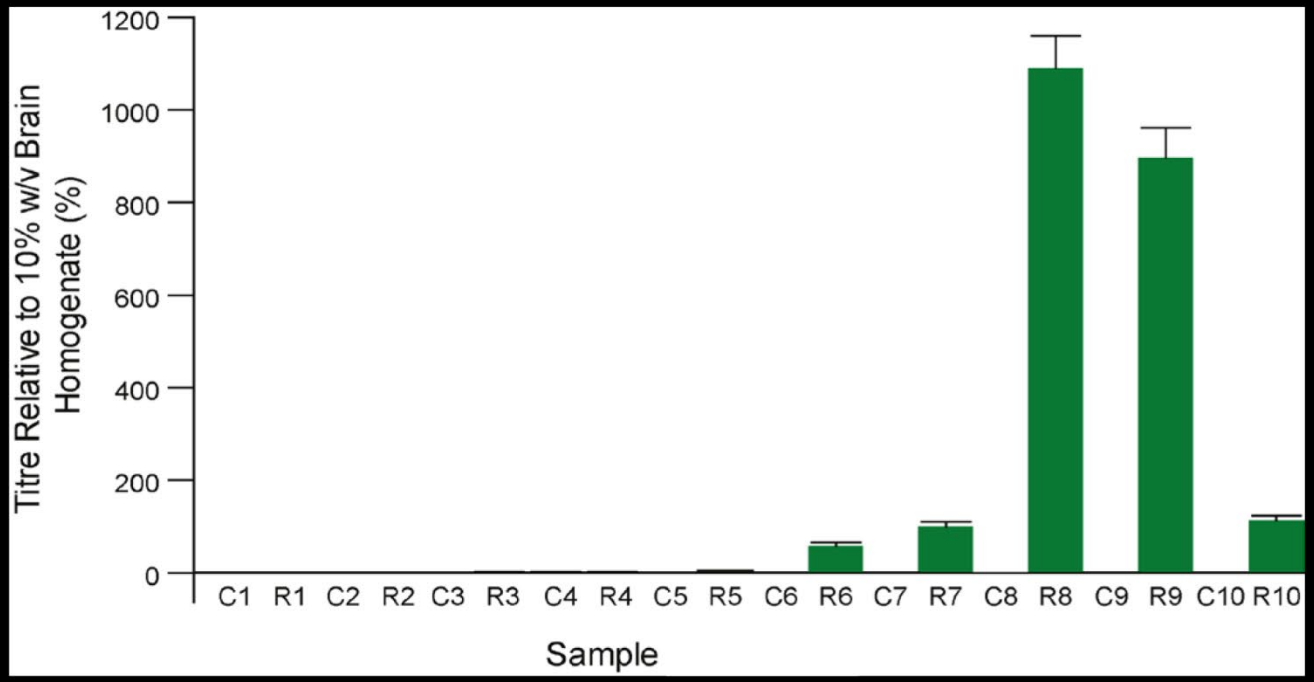




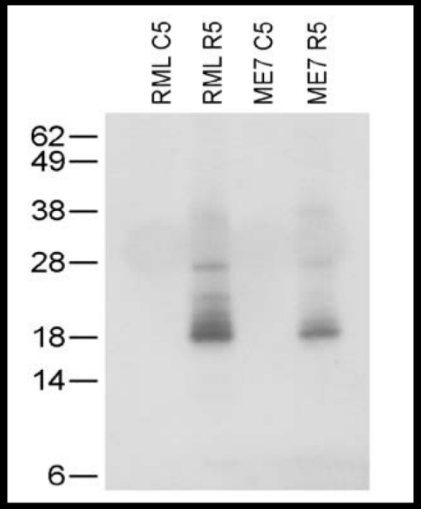
Klohn et al, PNAS 2003

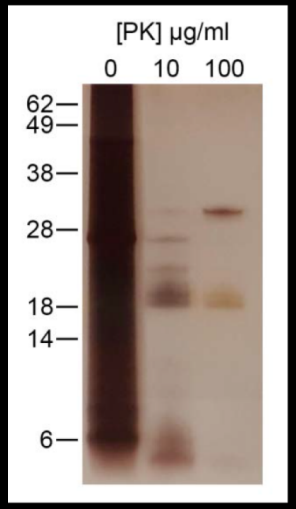
Determination of infectious titre by SCA

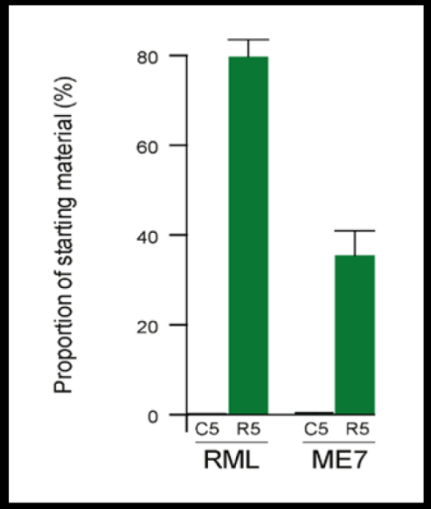


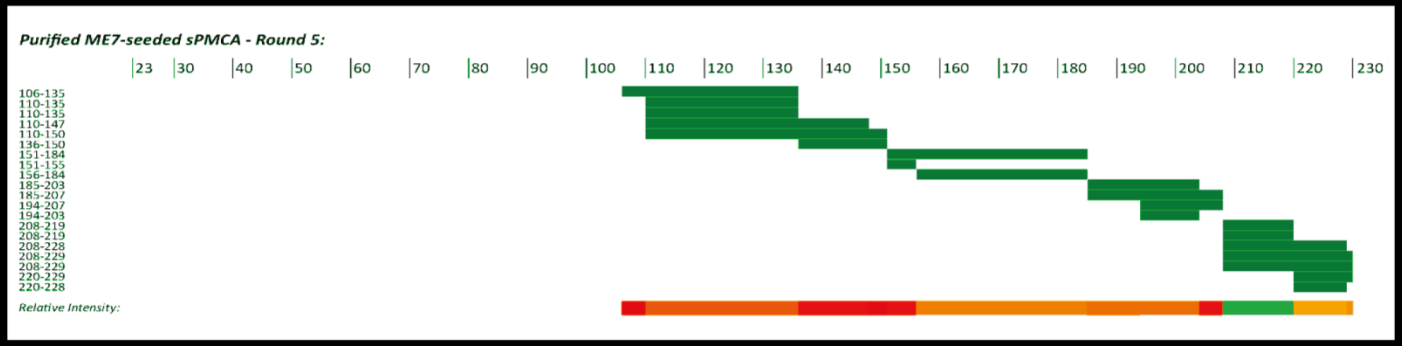


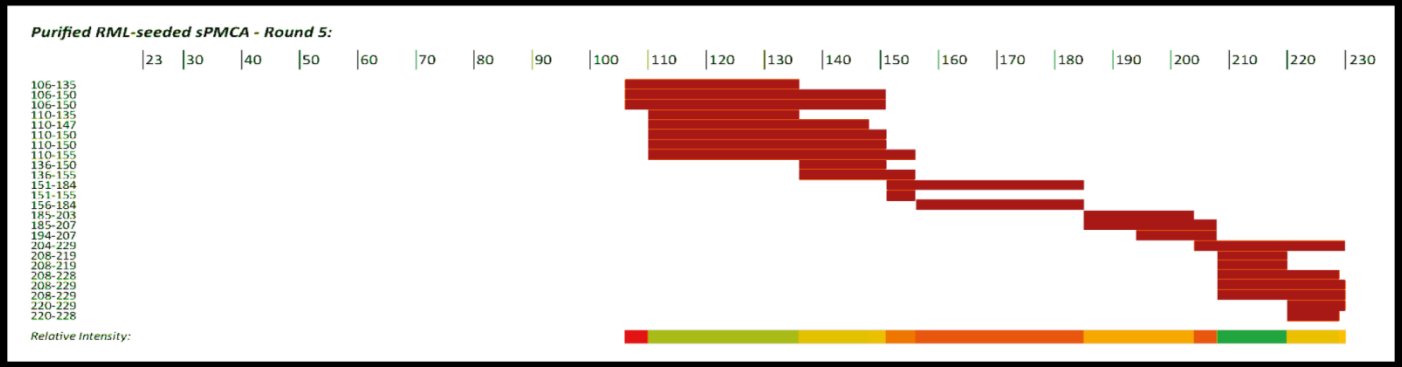
Purification and MS of SYPRIONS



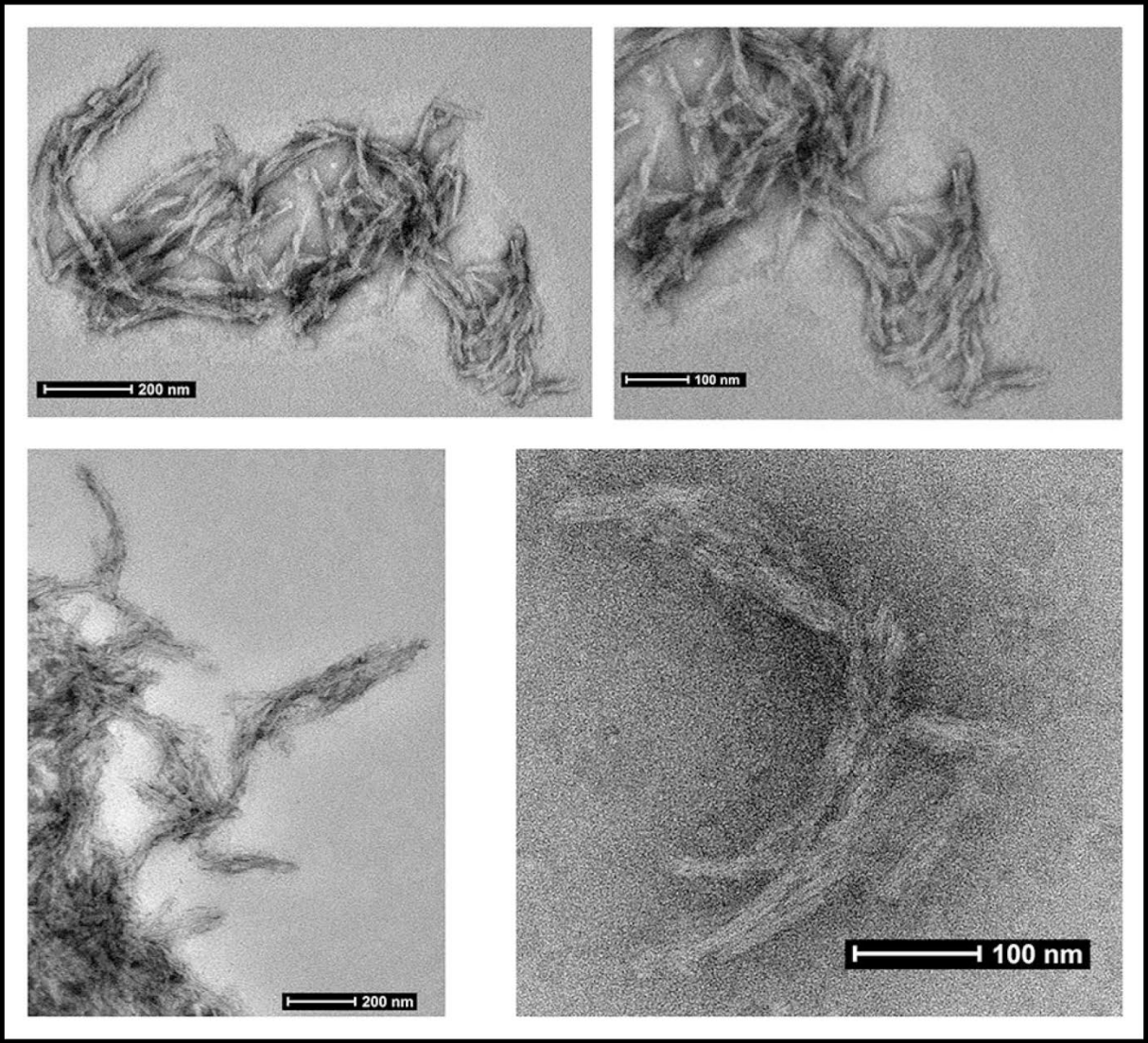




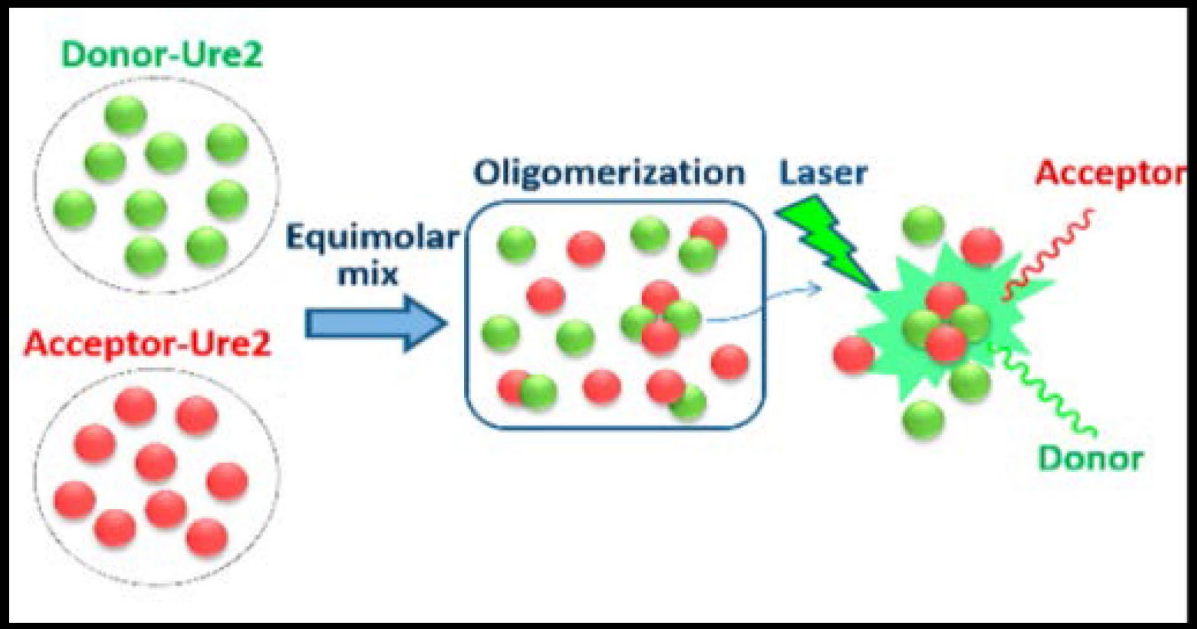


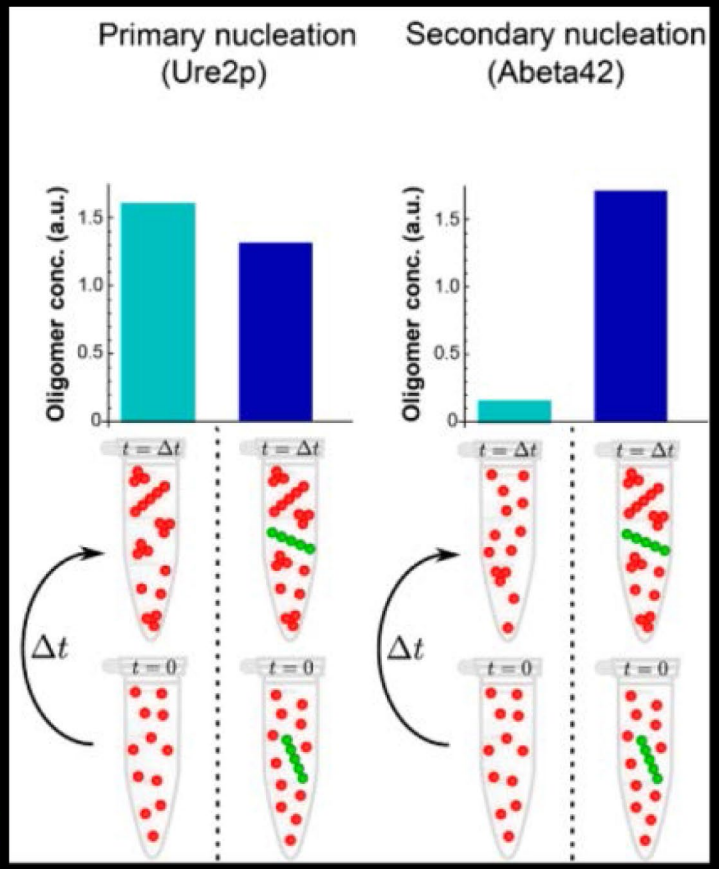


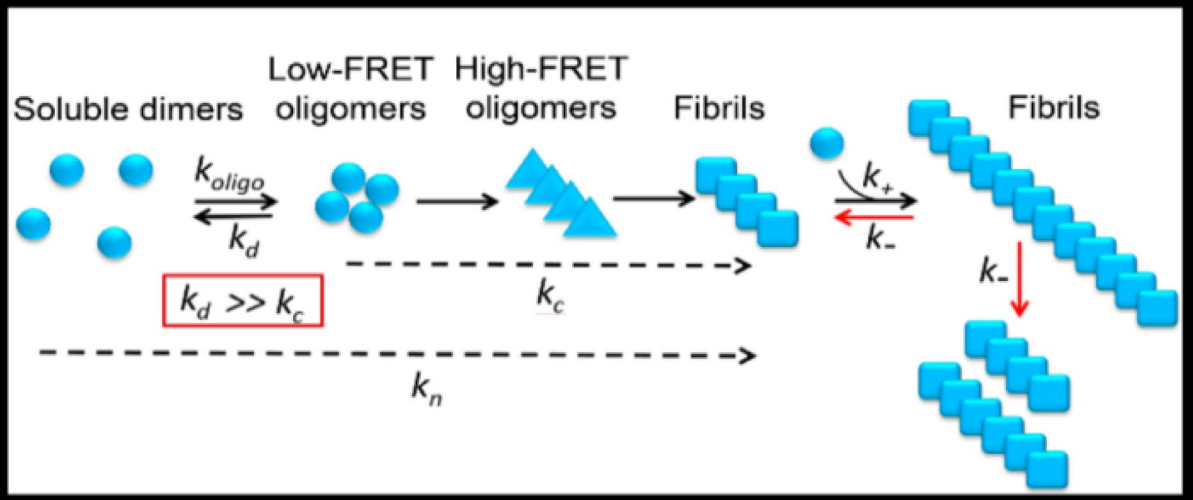
Negative stain EM confirmed SYPRIONS have a paired rod architecture in common with *ex vivo* material



RT-PMCA will enable sensitive, quantitation of prion replication rates

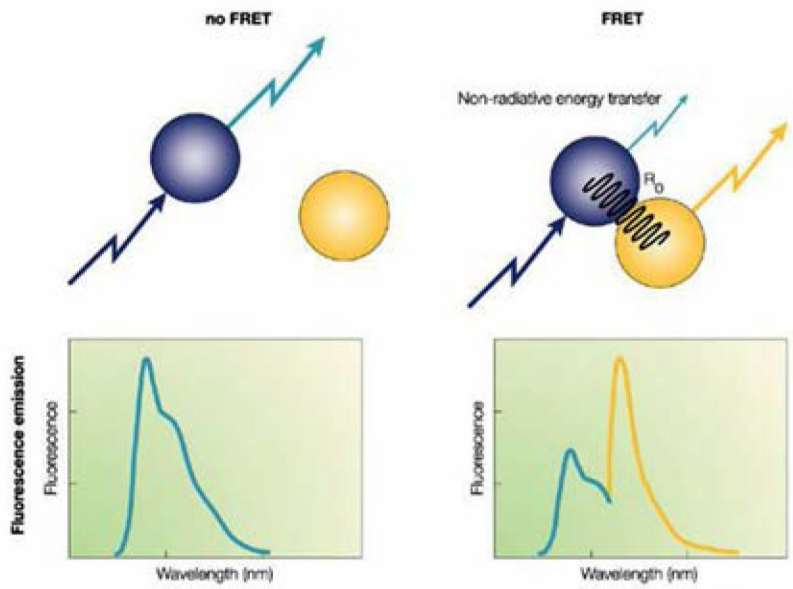






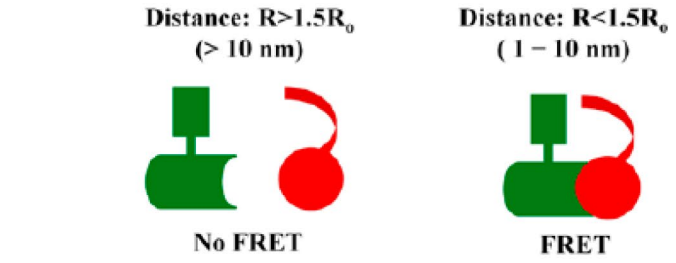
Yang et al, Journal of the American Chemical Society 2018

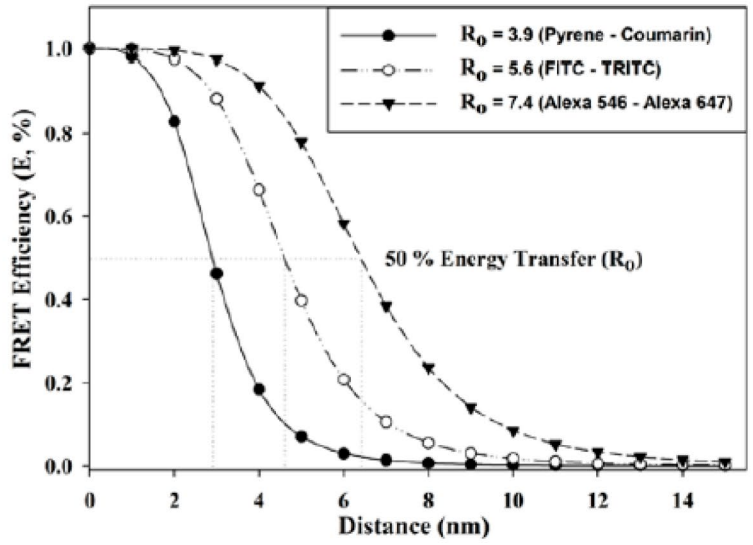
Förster resonance energy transfer (FRET)



$$E = \frac{1}{1 + (r/R_0)^6}$$

R_0 - Förster distance of the pair of donor and acceptor, i.e. the distance at which the energy transfer efficiency is 50%



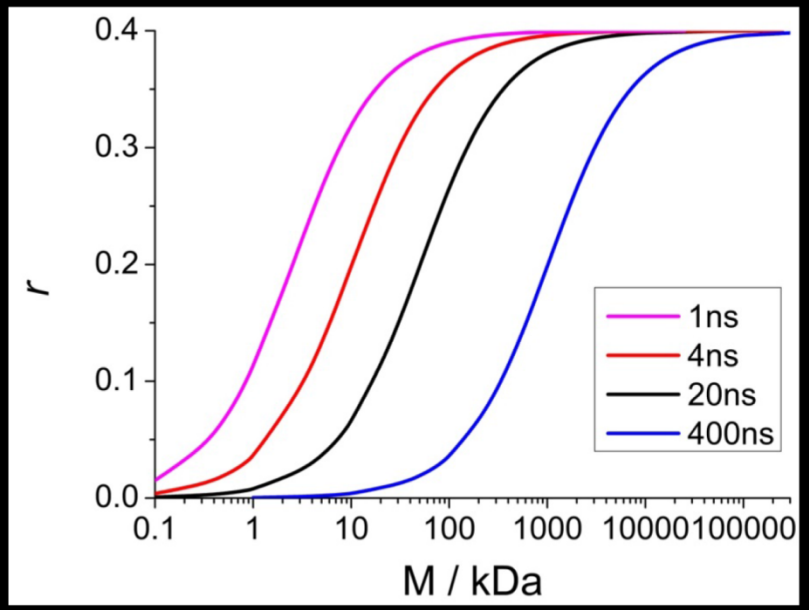


Fluorescence Anisotropy

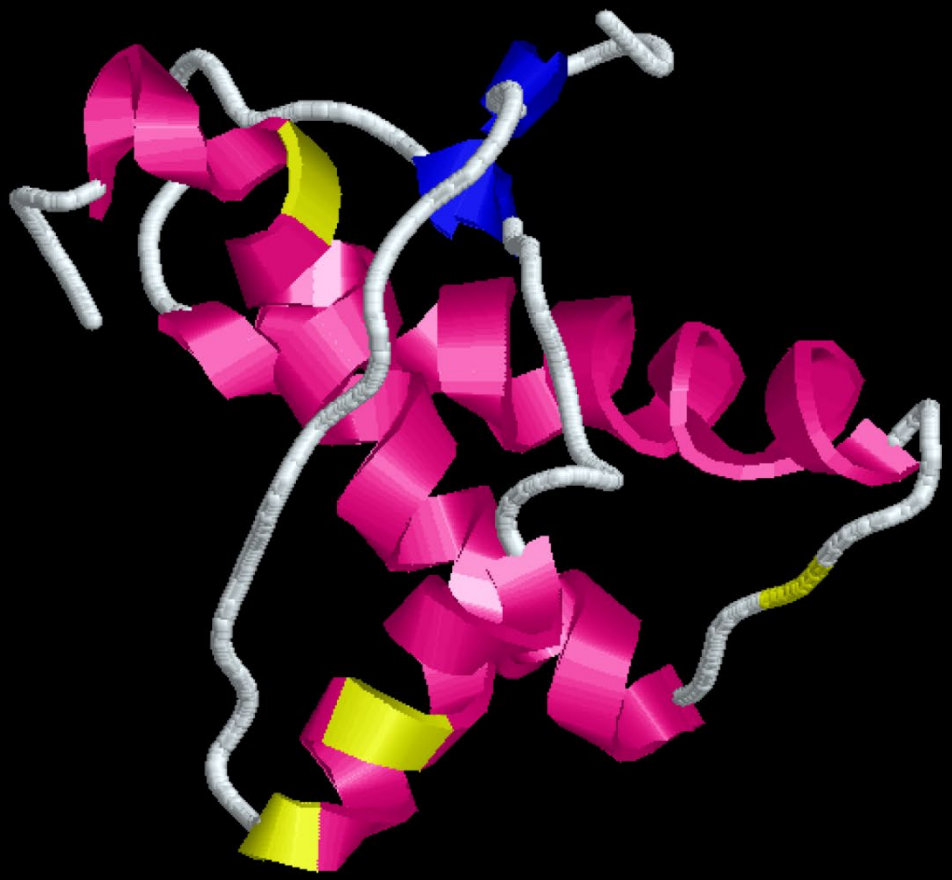
Described by the Perrin Equation where r is the observed anisotropy , r_0 is the intrinsic anisotropy of the fluorophore (increased following FRET which is inherently anisotropic), τ is the lifetime of the fluorophore and θ is the rotational correlation time of the fluorophore.

$$r = \frac{r_0}{1 + \tau/\theta}$$

Hence the slower the motion of the fluorophore (the larger it is), the greater the anisotropy.



Cysteine mutants for fluorescent labelling



W31C

W98C

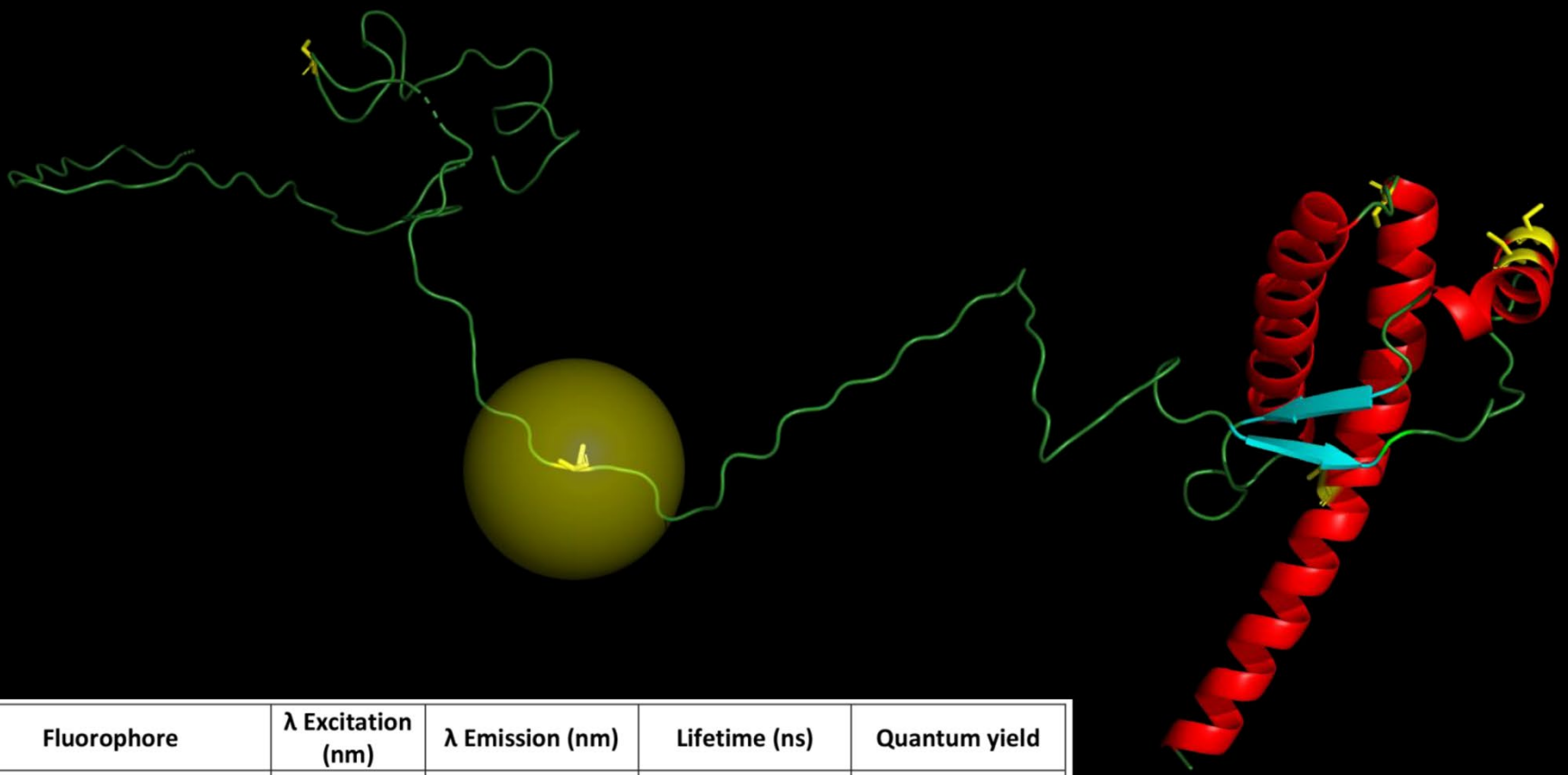
W144C

Y148C

F197C

Y217C

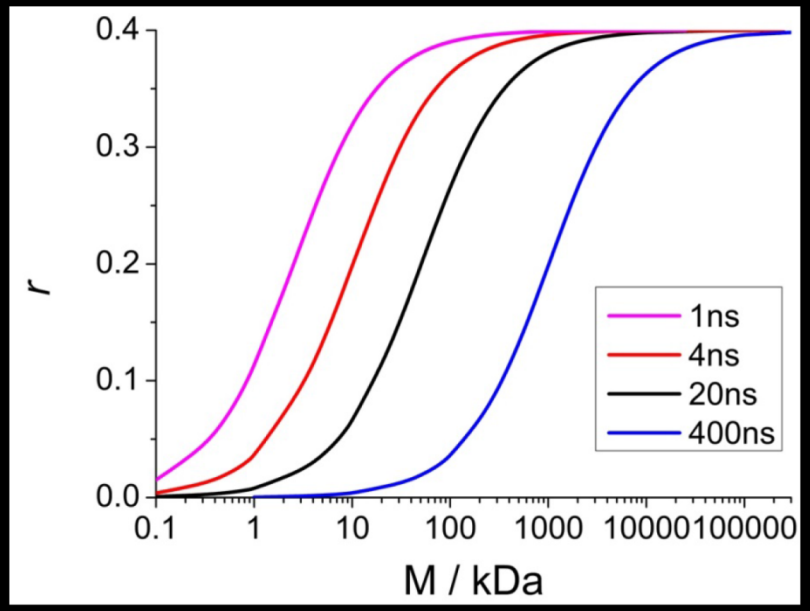
Förster radius of resonance energy transfer for Pyrene



Fluorophore	λ Excitation (nm)	λ Emission (nm)	Lifetime (ns)	Quantum yield
Tryptophan	285	340	2.5	0.12
Pyrene	338	375	300	0.32
DAC (dimethylamino coumarin)	383	463	3	0.73
Thioflavin T	412	482	0.001-0.3	Variable 0.0001-0.10
NBD (nitrobenzoxadiazole)	485	535	1-10	0.86

Are SYPRIONS concentrated enough for fluorescence detection ?

- Detection limit of fluoresescence < 1 pM
- Detection limit of FRET ~1nM
- Detection limit of RT-FRET (anisotropy increases following non-radiative transfer) ~2nM
- Size range for pyrene fluorescence lifetime (100kDa -100MDa)
- SYPRION concentration ~ 10ug/ml (~500nM monomer equivalent)



Summary

- We have generated high concentrations of synthetic prions that incorporate genetically modified prion proteins (PrP).
- We have made several genetically-modified PrPs that can be tagged with a variety of fluorescent molecules.
- We can use fluorescence to watch the growth of prion rods in real time during a PMCA reaction.
- Using fluorescence, resonance energy transfer and the asymmetry of emitted polarised light we can simultaneously monitor the loss of starting substrate and intermediate oligomers and prion rods as they form.
- The rate of growth of prion rods will be proportional to the number of prion seeds at the start of the reaction and will offer a rapid, sensitive and specific diagnostic measure.

MRC Prion Unit

UCL

Thank you !

CREUTZFELDT-JAKOB DISEASE FOUNDATION, INC.

Supporting Families Affected by Prion Disease

The Joanne (Jody) Atchison Memorial Grant

The Cheryl Molloy Memorial Grant

A Bequest from Beverly Nylund Huchala, in Memory of Peter Huchala

The Tom Stivison Memorial Grant

The Strides for CJD Grant