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Sensitive microscopic quantification of surface-bound prion infectivity for the assessment of surgical instrument decontamination procedures

T.J. Secker, R.C. Hervé*, C.W. Keevil

Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Southampton, UK

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SUMMARY

Background: Pathogenic prions (PrP^{Sc}) are amyloid-rich hydrophobic proteins which bind avidly to surgical surfaces and represent some of the most difficult targets during the reprocessing of reusable surgical instruments. In-vitro methods to amplify and detect the presence of otherwise undetectable prion contamination are available, but they do not measure associated infectivity. Most of these methods rely on the use of proteinase K, however this can lead to the loss of a substantial portion of PrP^{Sc}, potentially producing false negatives.
Aim: To develop a sensitive in-situ method without proteinase treatment for the dynamic quantification of amyloid accumulation in N2a #58 cells following 22L-prion infection from infected tissues and spiked stainless-steel surfaces.
Methods: We spiked cultures of N2a #58 cells with the 22L prion strain in solution or dried on stainless-steel wires and directly measured the accumulation of prion amyloid aggregates over several passages using highly sensitive fluorescence microscopy.
Findings: We demonstrated a 10-log dynamic range using our method to test residual prion infectivity, that was validated to show variable decontamination efficacy against prions from commercially available cleaning chemistries.
Conclusions: The new cell-based infectivity method presented here avoids partial or possibly total proteinase K digestion of PrP^{Sc} in samples for greater sensitivity, in addition to low cost, no ethical concerns, and adaptability to detect different prion strains. This method can be used to test cleaning chemistries' efficacy with greater sensitivity than measuring total residual proteins, which may not correlate with residual prion infectivity.

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* Corresponding author. Address: Environmental Healthcare Unit, School of Biological Sciences, Building 85, University of Southampton, Southampton, SO17 1BJ, UK. Tel.: +44 2381 205711
E-mail address: R.Herve@soton.ac.uk (R.C. Hervé).

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Introduction

Transmissible spongiform encephalopathies (TSEs) are rare, invariably fatal neurodegenerative conditions caused by the accumulation of an abnormally folded form of the prion protein (PrP^{Sc} or PrP^{res}) in the central nervous system of affected animals. These pathogenic prions are amyloid-rich, hydrophobic proteins which resist most standard reprocessing methods [1] and as such represent some of the most difficult targets for the reprocessing of reusable surgical instruments.

The classical, sporadic form of Creutzfeldt—Jacob Disease (CJD) in humans is caused by genetic mutations in the PRNP gene encoding for the prion protein and appears mostly confined to tissues within the central nervous system [2] with an estimated global prevalence of 1 per million. In contrast, variant CJD (vCJD) is caused by the cross-species horizontal transmission of prions from bovine spongiform encephalopathy (BSE)-infected cattle to humans through the food chain and has a wider tissue distribution. Variant CJD still represents the highest number of acquired CJD cases in the UK where it peaked in the year 2000 [3], with 178 recorded deaths to date since the start of surveillance in 1990 (data from the National Creutzfeldt—Jacob Disease Research and Surveillance Unit; NCJDRSU). Although the number of confirmed deaths from acquired CJD remains low, the estimated prevalence of asymptomatic vCJD carriers in the UK could be as high as 1/2000 [4]. A theoretical second wave of vCJD linked to extended incubation in genetically less susceptible patients exposed to BSE has failed to materialize to date, nevertheless the ostensibly long incubation period and the potential for disease transmission through infected blood [5—7] imply that many surgical procedures present a risk of iatrogenic transmission of vCJD. This has led to years of research and re-evaluation of clinical procedures to address the risk posed by prions in clinical settings.

Detection methods for biological markers associated with prion diseases are available, and we have developed staining methods to quantify prion amyloid aggregates on surgical surfaces [8]. However, no direct detection method is currently capable of assessing both infectivity and the presence of disease-associated PrP^{Sc} *in situ*. The 'gold standard' for prion infectivity studies has long relied on the infected wire implant animal bioassay. These are expensive, require ethical considerations, can take hundreds of days to obtain end-point data and are limited to using small-sized intracranial wire implants as models for complex surgical instruments.

Cell-based methods have been developed to directly detect prion infectivity post decontamination using model surgical surfaces (reviewed in [9,10]). The prion transmission within neuroblastoma cell lines has been studied from the late 1980s [11]. A method using a clone of scrapie N2a cells, denoted scN2a, showed susceptibility to infection from three different mouse-adapted prion scrapie strains: 139a, Chandler and 22L. However, these cells were not infected by the 87V and 22A strains [12], possibly due to differences in lineage and pathological profiles also reported *in vivo* [13]. Klohn *et al.* developed the standard scrapie cell assay (SSCA), further developed to form the scrapie cell endpoint assay (SCEPA) which involves the analysis of cells exposed to serial dilutions of prion strains [14—16]. The SCEPA assay to detect infectivity of prions absorbed on stainless-steel wires (standard steel-binding assay; SSBA), which also relies on immunodetection of PrP^{Sc}, was later used to assess the efficacy of various cleaning chemistries against prion infectivity [17].

However, these methods still rely on multiple biochemical techniques which may include proteinase K (PK) digestion and ex-situ immunodetection of PrP^{Sc} using Western blot or ELISA. There is evidence that up to 80% of PrP^{Sc} may be sensitive to PK digestion [18—21], and infectivity has been shown without any detectable PrP^{Sc} [22—26], suggesting that a number of insufficiently sensitive detection methods for PrP^{Sc} fail to correlate with actual residual prion infectivity.

The present study describes a cell-based infectivity assay that incorporates sensitive in-situ staining for the microscopy detection of prion infectivity and has the potential to be transposed to analyse a wide range of prion strains, using appropriately susceptible cell lines. Furthermore, this method can be used to assess surgical instrument decontamination procedures in relation to remaining prion infectivity using inoculated wires as the source of infection within the cells.

Materials and methods

Neuroblastoma cells

Neuroblastoma cell sub-clone (N2a#58), transfected with wild-type mouse PRNP α-cDNA to increase the expression of PrP^{c} within the cells, was kindly supplied by Prof. Sylvain Lehmann (Institut de Génétique Humaine, Montpellier, France) [27]. N2a#58 cells were cultured in sterile Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS; Invitrogen), 1% (v/v) 100× penicillin/streptomycin (pen/strep; Gibco, Invitrogen) and 1% (v/v) 100× GlutaMAX (Gibco, Invitrogen). Cultures were incubated in a humidified environment at 37°C and 5% CO_2 and media were replaced 24 h post seeding, then every two to three days following.

N2a#58 cells were seeded into 90-mm tissue culture dishes (Greiner Bio-One, UK) for experimental preparation. Cells were passaged by dislodging using a cell scraper at 90% confluency and split at a ratio of 1:50 into 35-mm dishes for infection studies.

Brain homogenates

Murine-scrapie 22L-infected brain homogenate was kindly supplied by Dr Ayodeji Asuni (School of Biological Sciences, University of Southampton). This was prepared from the brains of C57BL/6J mice inoculated via bilateral intra-hippocampal injections of 22L-infected homogenate. Animals were culled between 19 and 21 weeks (early symptomatic stage) and infected brains were collected and homogenized (10% w/v) in phosphate-buffered saline (PBS).

Murine normal brain homogenate (NBH; The Roslin Institute, University of Edinburgh) was prepared from brains dissected from adult C57BL mice homogenized (10% w/v) in PBS. Prior to inoculating the cells, the 22L-infected homogenate was partially purified to prevent brain material clumping and hindering microscopic amyloid detection and/or reducing cell viability, using a previously described method [28]. Briefly, brain homogenate was treated in lysis buffer (0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 0.5% (v/v) Triton X-100 and 50 mM Tris-HCl) for 20 min on ice, followed by 10 min centrifugation at

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17,000 *g*. The supernatant was made up to 500 μl in DMEM, sterile filtered through a 0.22-μm filter and further diluted to the required concentration.

Cell infection using prion-infected tissues

The N2a#58 cells from 90-mm dishes were split at a ratio of 1:50 as described above into four 35-mm dishes per condition. At this stage (P1) cells were subjected to NBH or dilutions of 10% 22L-infected brain homogenate, ranging from 10^{-4} to 10^{-10} in culture medium, to determine the sensitivity of our method for prion propagation over several passages, directly from infected brain material. Culture media was replaced after 24 h post-inoculation and every 48 h until confluency. Once confluent, the amyloid accumulation as a marker of prion propagation in cell cultures was detected in three of four dishes using Sudan black (SB)/thioflavin T (ThT) staining as described below, and cells from the fourth dish were split 1:20 into four new 35-mm dishes (P2). This was repeated at every subsequent passage until a loss in cell viability was observed (between passages 6 and 8 post infection).

Cell infection using prion contaminated surfaces

Surgical-grade 316 stainless-steel wires (0.16 mm diameter; Ormiston wire, UK) were cut to 5-mm lengths, soaked in acetone to remove any organic material, and then autoclaved in distilled water for 20 min at 121°C. Batches of 20 wires were inoculated in 10^{-4} to 10^{-10} dilutions of 10% (w/v) 22L-infected brain homogenate in DMEM media for infected samples or 10%

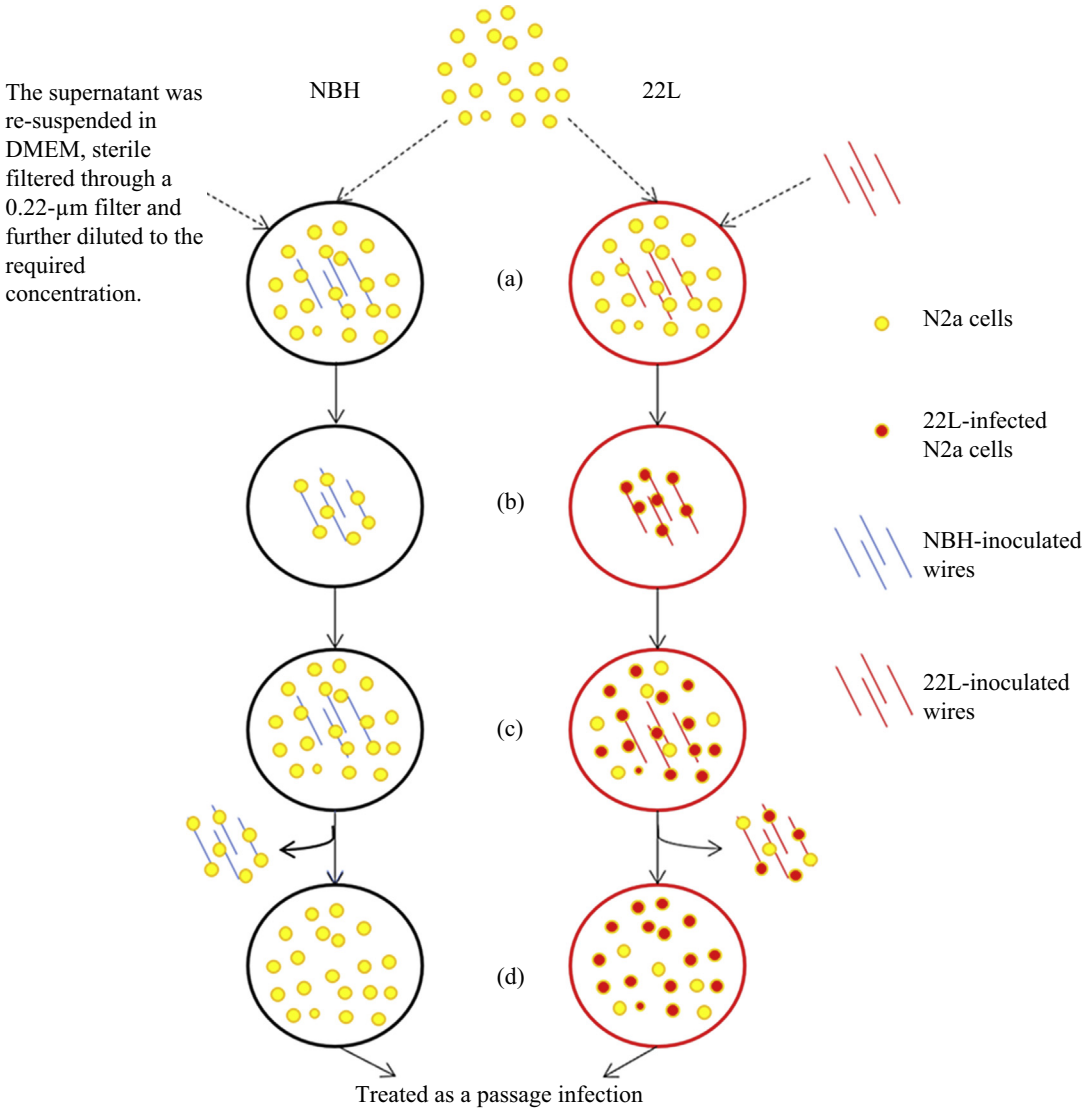

Figure 1. Schematic diagram showing the steps involved in initiating the N2a#58 wire infectivity assay. (a) Normal brain homogenate (NBH) or 22L inoculated wires were introduced to 35-mm tissue culture dishes, covered in N2a#58 cells, and cultured to confluency. (b) Wires coated in N2a cells were transferred to fresh 35-mm tissue culture dishes and again cultured to confluency. (c) The wires were removed and discarded, and the remaining cells were passaged and grown to confluency. (d) Passage 1 post wire removal: once confluent cells were treated as an N2a#58 passage infection study with Sudan black (SB)/thioflavin T (ThT) microscopic analysis and further passages.

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(w/v) NBH in DMEM media for uninfected controls. The wires were left inoculating for 2 h at room temperature and then removed and dried in separate filter-paper-covered Petri dishes for 2 h at 37°C.

Inoculated wires were placed in separate 35-mm cell culture dishes (as above), covered with 2 mL of pre-warmed DMEM medium containing N2a#58 cells split 1:50 from 90 mm culture dishes as described above. Cells were grown over the wires to confluency (~seven days of culture; Figure 1(a)) with media changes every 48–72 h. Once confluent the cell-covered, inoculated wires were removed and placed in 2 mL of fresh pre-warmed DMEM media in 35-mm dishes and the cells transferred with the wires were cultured to confluency (Figure 1(b) and (c)). Wires were then removed and discarded, the cells (considered at P1 here; Figure 1(d)) were then treated in the same way as the passage infection studies described above.

Wire decontamination studies

Wires (5 mm length) were prepared as described above and inoculated in 10% (w/v) 22L-brain homogenate for 2 h at room temperature. Once dried, the wires were treated using one of three commercially available cleaners according to the

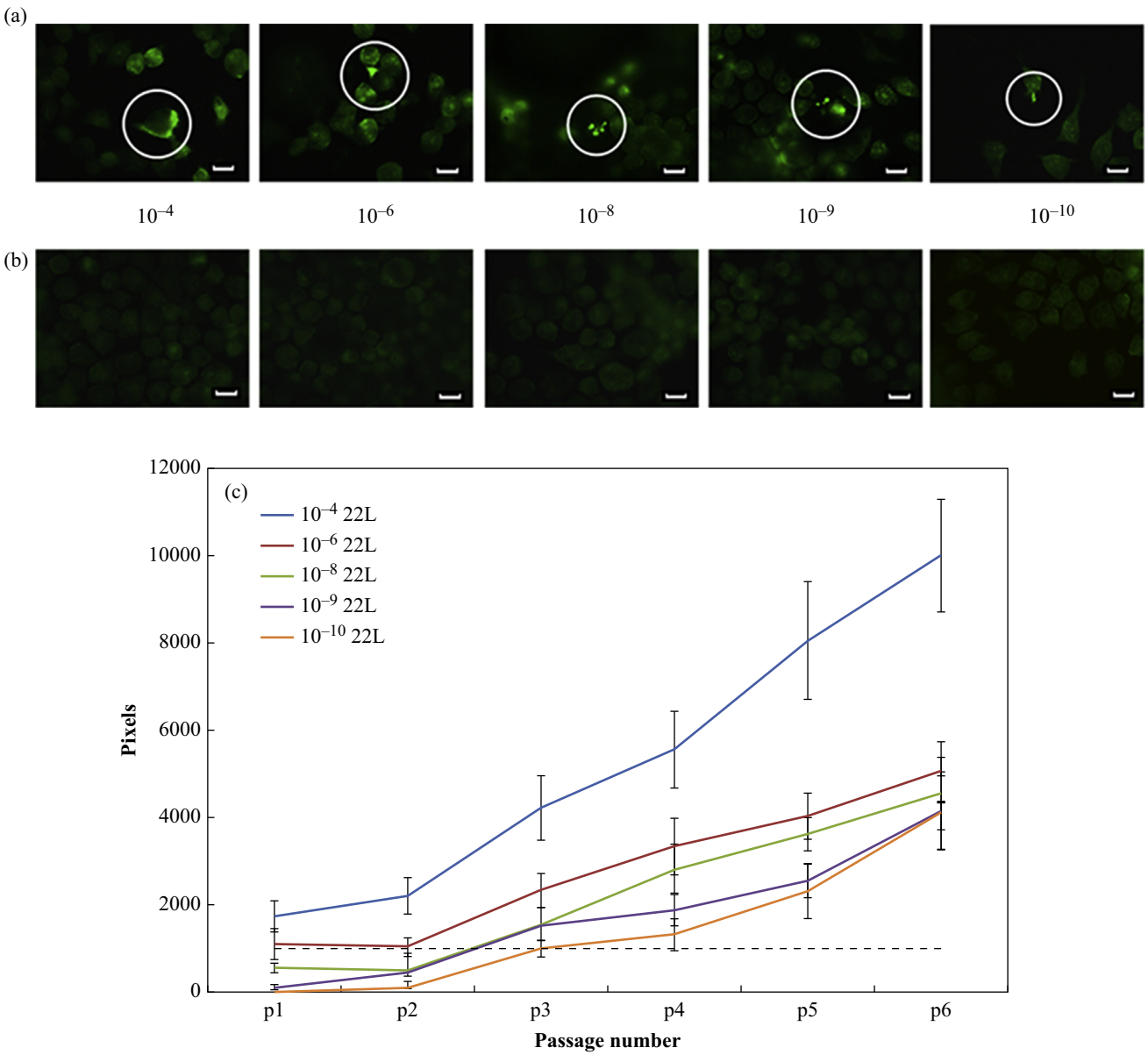

Figure 2. Example micrographs of Sudan black (SB)/thioflavin T (ThT) fluorescence taken from infected (a) and uninfected (b) N2a#58 cells. These images taken from samples at the penultimate passage show intracellular prion amyloid aggregates producing the characteristic Stokes shift of amyloid-bound ThT fluorescence (white rings) from 10^{-4} to 10^{-10} dilutions of 22L-infected brain homogenate. Scale bars = 10 μm. (c) ThT-positive signal per field of view over six passages every 10 days. Data are shown as means ± standard error of the mean ($N = 30$).

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manufacturers' recommendations; Cleaner 1 (Enzol, 20 mL/L, room temperature, 10 min, Johnson and Johnson); Cleaner 2 (Klenzyme, 8 mL/L, 43°C, 5 min, Steris©) and Cleaner 3 (Hamo 100, 8 mL/L, 43°C, 7 min, Steris©). To determine infectivity of residual prions after cleaning, decontaminated wires were analysed using the N2a#58/22L and SB/ThT detection method described below.

Prion amyloid staining within cells

The auto-fluorescent quenching properties of SB were examined for its suitability to suppress the autofluorescence associated with the cells. Prion-associated amyloid levels were detected in the cell culture samples using the amyloid-specific fluorophore ThT (excitation 405—450 nm and emission ≥475 nm) based on a protocol used on tissues described elsewhere [8,29,30], with the following modifications.

Culture medium was removed, and the cells were washed thoroughly in PBS (Gibco), then fixed for 6 min in 4% (v/v) paraformaldehyde in PBS (PFA; VWR). Cells were permeabilized in 0.1% (v/v) Triton X-100 in PBS for 10 min, followed by three PBS washes. The cells were stained with 0.3% (w/v) SB made up in 70% ethanol for 10 min. After SB staining, cells were washed in PBS and all cells were stained with 0.004% (w/v) ThT made up in 0.01 M hydrochloric acid for 15 min. After ThT staining, the cells were washed in 0.1% (v/v) acetic acid for 2 min to remove any non-specifically bound ThT, followed by washes in PBS and dH_2O. Cells were left in the final wash of dH_2O until microscopic analysis.

ThT/SYPRO ruby dual staining of contaminated wires

ThT (Sigma; 0.2 % w/v in 0.01 M hydrochloric acid) was applied on to samples for 10 min, followed by 0.1 % (v/v) acetic acid for 10 min, rinsed with PBS and then deionized water prior to adding SYPRO Ruby (SR; Invitrogen, UK) for 15 min, followed by three final washes in deionized water [8].

Nile red/ThT dual staining of contaminated wires

Nile red (NR)/ThT staining is an adaptation of the ThT/SR staining described above, where NR (Sigma; 5 mM w/v in DMSO) was used instead of SR to analyse total lipid instead of total protein. The NR staining preceded the ThT staining as the DMSO would quench the ThT signal.

Epifluorescence microscopy and image analysis

The fluorescent signal in stained samples was directly measured using episcopic differential interference contrast microscopy coupled with epifluorescence (EDIC/EF; Best Scientific, Swindon, UK) [31]. The ThT signal within the monolayer cultures was observed using 1000× total magnification across 10 random areas from three stained dishes per condition (excitation 405—450, emission >475 nm, Nikon). Images were captured using a CCD colour camera (Roper Industries, UK) with identical camera detection settings for all samples after the threshold was optimized to exclude low levels of background in the negative control samples. Images were analysed using Image J software to quantify positive ThT signal (above negative control), expressed in pixels per field of view or mm^2 for cell cultures or wires, respectively. Based on our observations, a sustained increase beyond 1000 pixels per field of view was considered as confirmed infection, to exclude areas of increased background fluorescence associated with the tumour-spheroid production of N2a cells.

EDIC/EF microscopy was also used for the analysis of the ThT/SR and NR/ThT staining of the inoculated wires pre- and post-decontamination. Scans of the contaminated areas were acquired at ×100 total magnification visualizing the SR or NR signal (excitation: 470 nm; emission: 618 nm) and ThT signal (as above). Captured images were analysed using Image J software and SR-positive signal was standardized to the surface areas of the wires and reported in pg/mm^2 BSA equivalent for the quantification of residual proteinaceous contamination, as described previously [32]. In the absence of a lipid standard results were expressed as positive $pixels/mm^2$.

Statistics

Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by the Games—Howell post-hoc test to determine the significance between the different samples (IBM SPSS). A value of $P \leq 0.05$ was considered significant.

Results

Infectivity titer from 22L-infected brain homogenate dilutions within the N2a#58 cells

The incorporation of SB into the method efficiently quenched lipid and lipofuscin autofluorescence background signal. Lowering the initial 22L infectious dose led to proportionately lower levels of ThT-positive signal measured *in situ* within the imaged cells compared with the highest 22L dose infections at subsequent passages (Figure 2, Table I). The highest 10^{-10} dilution required four cell passages before showing a significant increase compared with the negative control, though this signal difference consistently increased through subsequent passages, confirming infection.

Sensitivity of the SB/ThT staining technique to detect amyloid accumulation in N2a #58 cells post contact with 22L-inoculated wires

As with homogenates directly applied to cells, a dose-dependent increase in ThT signal was measured in the N2a cells through seven cell passages post removal of the inoculated wires. The cells that had been in contact with the 10^{-4} 22L-inoculated wires demonstrated a rapid increase in amyloid accumulation when compared with the lower dose infections. Here again, when compared with the negative control reference, the 10^{-10} 22L-infected cells only showed a significant increase in ThT signal after four passages post wire removal (Table I) and a further passage was required to exceed the 1000 pixels threshold confirming transmission and accumulation of PrP^{Sc} (Figure 3).

Validation of the N2a#58 wire infectivity method for the detection of residual prion infectivity post decontamination

All three commercially available cleaning chemistries demonstrated significant removal of total protein, amyloid,

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Table I
N2a#58 passage number after initial 22L infection and statistical significance of thioflavin T (ThT) signal at each passage compared with the uninfected controls.

Experiment	Sample	N2a#58 passage no. after initial 22L infection and significance of ThT signal at each passage compared with controls						
		P1	P2	P3	P4	P5	P6	P7
22L Brain homogenate titre infectivity (Figure 2)	10^{-4} 22L	***	***	***	***	***	***	
	10^{-6} 22L	—	—	***	***	***	***	
	10^{-8} 22L	—	—	**	***	***	***	
	10^{-9} 22L	—	—	**	***	***	***	
	10^{-10} 22L	—	—	—	*	*	***	
22L inoculated wire titre infectivity (Figure 3)	10^{-4} 22L	**	***	***	***	***	***	***
	10^{-6} 22L	—	***	***	***	***	***	***
	10^{-8} 22L	—	—	**	***	**	***	***
	10^{-9} 22L	—	—	***	***	***	***	***
	10^{-10} 22L	—	—	—	**	**	**	***
22L infectivity post cleaning (Figure 4)	+ve Control	***	**	***	***	***	***	***
	Cleaner 1	—	—	—	**	***	***	***
	Cleaner 2	—	—	—	**	***	***	***
	Cleaner 3	—	—	—	—	—	—	—

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; —, no significant difference.

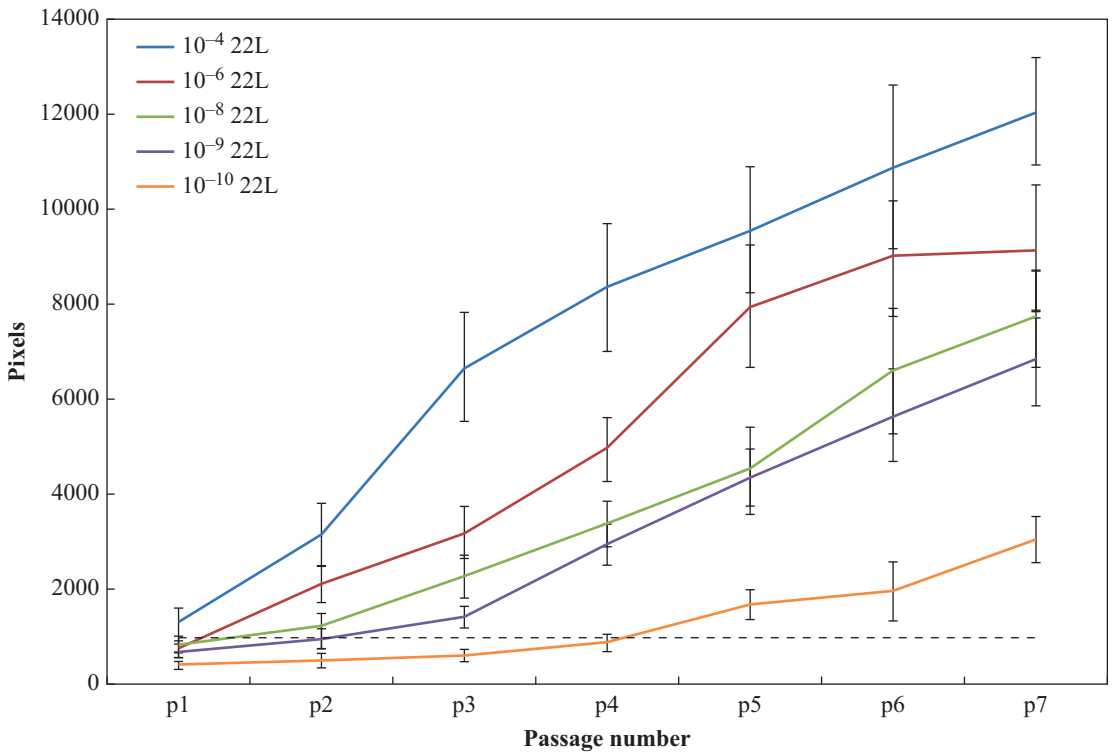

Figure 3. Profiles showing the dynamic evolution of Sudan black (SB)/thioflavin T (ThT)-positive signal per field of view observed within the N2a#58 cells infected using stainless-steel wires inoculated with dilutions of 22L-infected brain homogenate ranging from 10^{-4} to 10^{-10}. Data shows means ± standard error of the mean ($N = 20$).

and lipids from the inoculated wires, although residues were easily measured using EDIC/EF. Cleaners 1 and 2 achieved lower levels of residual proteins compared to Cleaner 3, however Cleaner 3 was the most efficacious against lipid contamination (Figure 4(a), (b)). The levels of residual amyloid (between 80 and 250 pg/mm^2) were similar between the three chemistries tested. From the positive control wires, an initial decrease in intracellular ThT signal was observed after passage 1, due to the high initial infectious dose being diluted, then the usual increase in ThT-positive signal was measured over the

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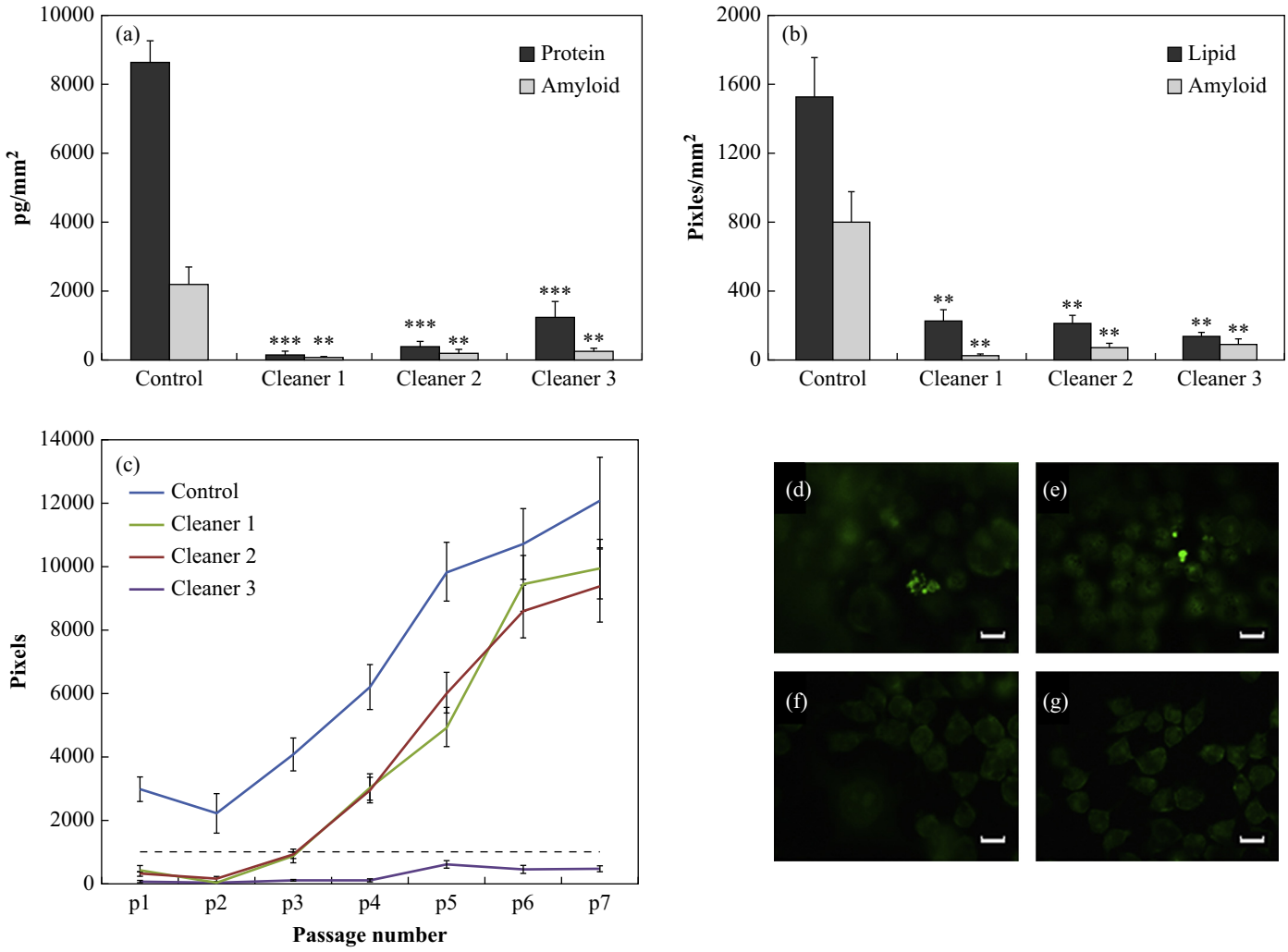

Figure 4. Total protein and prion amyloid contamination (a) and lipid contamination compared with prion amyloid contamination (b) measured pre- (control) and post-decontamination with Cleaner 1, Cleaner 2 or Cleaner 3, on surgical stainless-steel wires inoculated with 22L-infected brain homogenate (10% w/v; $N = 6–15$ wires). (c) Corresponding profiles of Sudan black (SB)/thioflavin T (ThT)-positive staining in N2a#58 cells (per field of view) over seven passages post contact with 22L-spiked surgical stainless-steel wires as above. Data shows means ± standard error of the mean ($N = 50$). ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared with the positive controls. Representative micrographs show SB/ThT staining in N2a#58 cells after seven passages following contact with 22L-inoculated surgical stainless-steel wire controls (d), post decontamination with Cleaner 2 (e), Cleaner 3 (f) and uninfected normal brain homogenate (NBH) controls (g). Scale bars = 10 μm.

subsequent passages (Figure 4(c), Table I). After a lag phase over the first two passages post wire removal, the N2a #58 cells that had been in contact with 22L-inoculated wires decontaminated with Cleaner 1 and Cleaner 2 both showed similar amyloid accumulation profiles from passages 3–7 (21–49 days) post wire removal (Figure 4(c), Table I). Figure 4(d) and (e) show ThT-positive plaques at the seventh passage (49 days post wire removal) in the positive control and with Cleaner 2, respectively. Figure 4(f) and (g) show the weak background ThT fluorescence in uninfected control cells and levels visible at passage 7 post Cleaner 3, respectively, which remained below our set threshold.

Discussion

It is important to use a cell line that will amplify the prion infection to a level at which the propagation is at a steady state but not engulfing or killing the cells [16,33]. Therefore, we chose the 22L prion strain and N2a #58 cells as an established prion infectivity model [12,27] to develop and determine the sensitivity of our novel staining procedure in relation to prion decontamination. The absence of PK digestion ensures that the entirety of the disease-associated material remains present, contributing to increased sensitivity. Direct observation of amyloid aggregates using ThT has previously shown at least 2-log greater sensitivity when compared with indirect Western Blot detection of PK-resistant PrP^{Sc} [8,29,30,34], and ThT is also used in in-vitro amplification assays measuring amyloid aggregation [35–37].

Our N2a#58/22L SB/ThT assay was successfully used to detect in-situ residual infectivity on surgical surfaces and was validated by comparing the infectivity remaining on 22L-inoculated wires post decontamination with three commercial cleaning chemistries. Accordingly, Cleaners 1 and 2 (enzymatic) both

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demonstrated an apparent reduction (~2.5 log) in infectivity, whereas Cleaner 3 (alkali) produced an approximate 7.9 log reduction in infectivity. Chemical residues potentially present on treated wires never affected cell viability (probably due to the relatively high dilution factor once placed within cell cultures medium) and are unlikely to have affected the staining after wires were removed and several medium replacements. These results are comparable with those reported in the hamster 263K model [38,39]. The higher log reduction in infectivity measured in the N2a#58/22L SB/THT method, compared with animal bioassays, is probably due to the higher sensitivity of this method (10 log compared with 7 log in the animal bioassays). This method could also be applied to detect other prion strains (particularly CJD or vCJD) from surgical surfaces or possibly blood samples using appropriate cell lines, rather than experimental models which may exhibit different responses to decontamination protocols [40–42]. Although not applicable as a routine surveillance tool, the N2a#58/22L SB/THT method offers an ethical alternative to assess decontamination protocols in the laboratory, and could be developed to test instruments with different surface types and configurations suspected of harbouring prions, which is not possible in animal bioassays. Post contamination conditions such as drying times could also be analysed with regard to residual infectivity, as drying conditions have been shown to vastly affect decontamination efficacy and prion attachment [43,44]. Finally, our method shows that decontamination efficacy, often judged by total protein removal, does not always correlate with residual infectivity of prion-infected soils.

In conclusion, the present study demonstrates a highly sensitive, cost-efficient alternative to animal bioassays, utilizing prion-associated amyloid specific fluorescent staining as opposed to immuno-labelling, without the need to PK digest the sample and which also permits the observation of prion propagation *in situ* within the culture system.

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Author contributions

T.J.S. carried out laboratory experimentation, data analysis and interpretation, participated in the design of the study, and drafted the manuscript; R.C.H. helped with experimental design, development, data analysis and interpretation, and finalizing the manuscript; C.W.K. conceived of the work, obtained the funding, coordinated the study, and helped with drafting the manuscript. All authors gave final approval for publication.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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