

Application of high-throughput, capillary-based Western analysis to modulated cleavage of the cellular prion protein

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Andrew R. Castle^{†§}, Nathalie Daude[‡], Sabine Gilch[¶], and  David Westaway^{†§||1}

From the [†]Centre for Prions and Protein Folding Diseases, University of Alberta, Edmonton, Alberta T6G 2M8, Canada [‡]Department of Medicine, University of Alberta, Edmonton, Alberta T6G 2G3, Canada; [¶]Department of Ecosystem and Public Health, Calgary Prion Research Unit, Faculty of Veterinary Medicine and Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta T2N 1N4, Canada, and ^{||}Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2R3, Canada

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The cellular prion protein (PrP^C) is a glycoprotein that is processed through several proteolytic pathways. Modulators of PrP^C proteolysis are of interest because full-length PrP^C and its cleavage fragments differ in their propensity to misfold, a process that plays a key role in the pathogenesis of prion diseases. PrP^C may also act as a receptor for neurotoxic, oligomeric species of other proteins that are linked to neurodegeneration. Importantly, the PrP^C C-terminal fragment C1 does not contain the reported binding sites for these oligomers. Western blotting would be a simple end point detection method for cell-based screening of compound libraries for effects on PrP^C proteolysis or overall expression level. However, traditional Western blotting methods provide unreliable quantification and have only low throughput. Consequently, we explored capillary-based Western technology as a potential alternative; we believe that this study is the first to report analysis of PrP^C using such an approach. We successfully optimized the detection and quantification of the deglycosylated forms of full-length PrP^C and its C-terminal cleavage fragments C1 and C2, including simultaneous quantification of β -tubulin levels to control for loading error. We also developed and tested a method for performing all cell culture, lysis, and deglycosylation steps in 96-well microplates prior to capillary Western analysis. These advances represent steps along the way to the development of an automated, high-throughput screening pipeline to identify modulators of PrP^C expression levels or proteolysis.

The cellular prion protein (PrP^C)² is a glycoprotein of ~208 amino acids that predominantly resides on the outer face of the

cell membrane, where it is attached via a glycosphosphatidylinositol (GPI) anchor. Full-length (FL) PrP^C is subject to several proteolytic processing pathways, including α -cleavage, which produces the C-terminal fragment C1 and the N-terminal fragment N1. α -Cleavage is generally thought to occur between residues 110 and 111 (human numbering) (1, 2), although a recent study using recombinant prion protein (recPrP) suggested that there are multiple cleavage sites in the 110–121 region (3). The rarer β -cleavage takes place within the metal ion-binding octapeptide repeat domain of PrP^C and results in the production of C2 and N2 fragments (3, 4). N1 and N2 are thought to be released from the cell, whereas C1 and C2 can be detected in cell lysates (5).

Physiological processing of PrP^C is of interest largely because misfolded prion protein, known as PrP^{Sc}, plays a key role in the pathogenesis of the fatal neurodegenerative disorders known as prion diseases. Importantly, C1 is resistant to the misfolding process and appears to be a dominant-negative inhibitor of PrP^{Sc} formation (6, 7). The longer fragment, C2, seems to retain the ability to misfold (8) and may also be resistant to subsequent cleavage to C1 at the “ α ” site (9). Therefore, compounds that enhance α -cleavage or inhibit β -cleavage or suppress overall PrP^C expression would be useful therapeutic agents for prion diseases. Furthermore, soluble β -amyloid and α -synuclein oligomers may elicit neurotoxicity partly via binding to PrP^C (10–12). Given that such oligomers are linked to the neurodegeneration observed in Alzheimer’s and Parkinson’s diseases, respectively, and that the putative PrP^C binding sites are all absent in C1, compounds that modify PrP^C expression or proteolysis may also be protective against these disorders.

The relatively low steady-state levels of the C2 fragment can complicate analysis of β -cleavage events. However, a recent study from our laboratory showed that a mutant PrP^C (known as “S3”) with several amino acid substitutions within its octapeptide repeat domain displays accentuated β -cleavage in certain cell types (13). Cells expressing S3 PrP^C can therefore be used to screen compounds for their effects on C1, C2, and FL PrP^C levels simultaneously. Even so, our ability to perform such screens has been hampered by the low-throughput and semiquantitative nature of the most straightforward detection technique, Western blotting. One novel technology with the potential to solve this issue is a microfluidic, capillary Western assay analysis system called Simple Western. In this approach, protein samples are size-separated by capillary electrophoresis and immobilized by UV-cross-link-

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¹ To whom correspondence should be addressed: Centre for Prions and Protein Folding Diseases, 2-04 Brain and Aging Research Bldg., University of Alberta, Edmonton, AB T6G 2M8, Canada. Tel.: 780-492-9024; Fax: 780-492-9352; E-mail: david.westaway@ualberta.ca.

² The abbreviations used are: PrP^C, cellular prion protein; GPI, glycosphosphatidylinositol; FL, full-length; recPrP, recombinant prion protein; PNGase F, peptide-N-glycosidase F; MW, molecular weight; %CV, percentage coefficient of variation; trxA, thioredoxin A; HRP, horseradish peroxidase; TBS, tris-buffered saline.

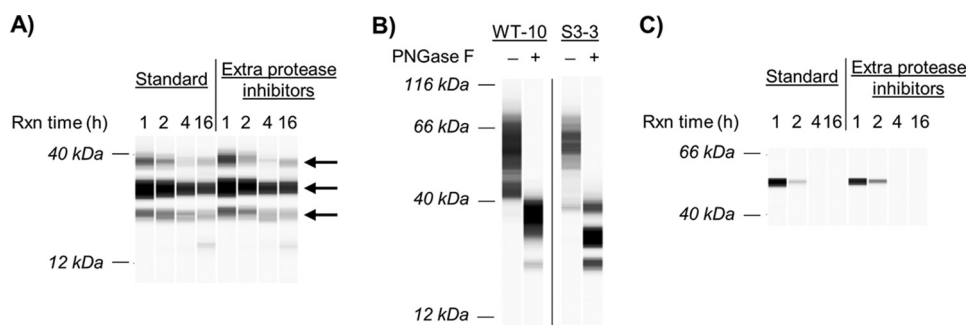


Figure 1. Prolonged incubations of cell lysates with PNGase F result in protein degradation. A, capillary Western lane view image showing PrP^C immunodetection in S3-3 RK13 cell lysates exposed to PNGase F for a range of incubation periods either with or without higher concentrations of protease inhibitors (arrows from top to bottom indicate FL PrP^C, C2, and C1). B, capillary Western lane view images comparing PrP^C immunodetection patterns in PNGase F-treated and untreated WT-10 and S3-3 RK13 cell lysates (the dividing line indicates that the data were from separate assays). C, capillary Western lane view image showing β -tubulin immunodetection in S3-3 RK13 cell lysates exposed to PNGase F for a range of incubation periods either with or without higher concentrations of protease inhibitors. Rxn, reaction.

ing. Antibody binding, wash steps, and subsequent chemiluminescence-based detection also take place on the capillaries via automated sequential delivery of assay reagents. Processing time is reduced to a few hours, which greatly increases throughput. In addition, the mostly hands-free nature of this system offers the potential for more consistent quantification than traditional Western blotting. Furthermore, sample loading volumes are approximately five times smaller than in gel electrophoresis, which reduces reagent use, helps preserve sample stocks, and offers the potential for automating manual handling steps that precede Western blot analysis. Capillary Western technology has already been put to use in the neurodegeneration field, including for analyzing the expression of the microtubule-associated protein Tau in brain homogenates (14) and detecting biomarkers to diagnose neurodegenerative diseases (15, 16).

In this manuscript, we outline our progress in optimizing the detection and quantification of the GPI-linked glycoprotein PrP^C and its cleavage fragments using capillary Western technology. We also report on work to optimize the deglycosylation of PrP^C in cell lysates, including the development of an “in-plate” format to process samples for capillary Western analysis.

Results

Optimization of PrP^C deglycosylation by peptide-N-glycosidase F

Most PrP^C molecules are posttranslationally modified by the addition of two N-linked glycans, resulting in smeared bands on Western blots. This affects the accuracy of quantification and makes it difficult to isolate bands corresponding to the different forms of PrP^C. Therefore, N-linked glycans are usually removed by treatment with the enzyme peptide-N-glycosidase F (PNGase F) when quantification is required. To take full advantage of the potential for enhanced quantification afforded by capillary Western analysis, we decided to identify the optimum combination of PNGase F concentration and incubation time. In our study, we treated a lysate of RK13 cells stably expressing S3 PrP^C (clone 3, hereafter referred to as S3-3) with 5 units/ μ l PNGase F for incubation periods of 1–16 h at 37 °C. The PrP^C C-terminal antibody Sha31 (17) was used to detect PrP^C, as was the case for all experiments reported in this manuscript unless stated otherwise. PrP^C signals decreased with

increasing PNGase F incubation time (Fig. 1A), presumably because of protein degradation. Surprisingly, this effect was not blocked by the addition of fresh protease inhibitors at the manufacturer’s recommended concentration prior to incubation with PNGase F (Fig. 1A). However, we saw no need to pursue this unexpected result further because a 1-h incubation time was sufficient for complete deglycosylation of PrP^C, as demonstrated by the three well-defined bands in the far right lane of Fig. 1B, which represent C1, C2, and FL PrP^C (smallest to largest). In contrast, PrP^C immunostaining in an untreated S3-3 RK13 lysate presented as a smear covering a wide range of molecular weights (MWs). Fig. 1B also shows that a 1-h incubation led to complete deglycosylation of PrP^C in a lysate of RK13 cells stably expressing WT murine PrP^C (clone 10, hereafter referred to as WT-10).

The Simple Western system allows multiple targets to be detected in a single capillary (“multiplexing”) as long as the peaks obtained are sufficiently separated by size. Therefore, we investigated loading control proteins that could be analyzed alongside deglycosylated PrP^C. β -Tubulin was a potential candidate because of its apparent MW of ~55 kDa following separation by SDS-PAGE. We tested β -tubulin stability over the same range of PNGase F incubation periods and found that it was considerably less stable than PrP^C (Fig. 1C). β -Tubulin immunodetection was greatly reduced after 2-h incubation compared with 1 h and was completely absent following longer incubations. Again, the effect was not blocked by increasing the concentrations of protease inhibitors. Together, these data suggested that incubating cell lysates for 1 h with 5 units/ μ l PNGase F was the best approach.

Differential sizing of full-length PrP^C and its cleavage fragments in capillary and traditional Western assays

During our initial experiments with the capillary Western system, we observed that PrP^C and its cleavage fragments appeared to migrate more slowly than in standard SDS-PAGE-based separation. Fig. 2A displays the PrP^C immunostaining pattern in WT-10 and S3-3 RK13 cell lysates from a traditional Western blot in the absence of deglycosylation. Despite the smeared staining pattern, the shift to higher apparent MWs with the capillary system is obvious (Fig. 2B). The difference was still apparent after PNGase F treatment; FL PrP^C was

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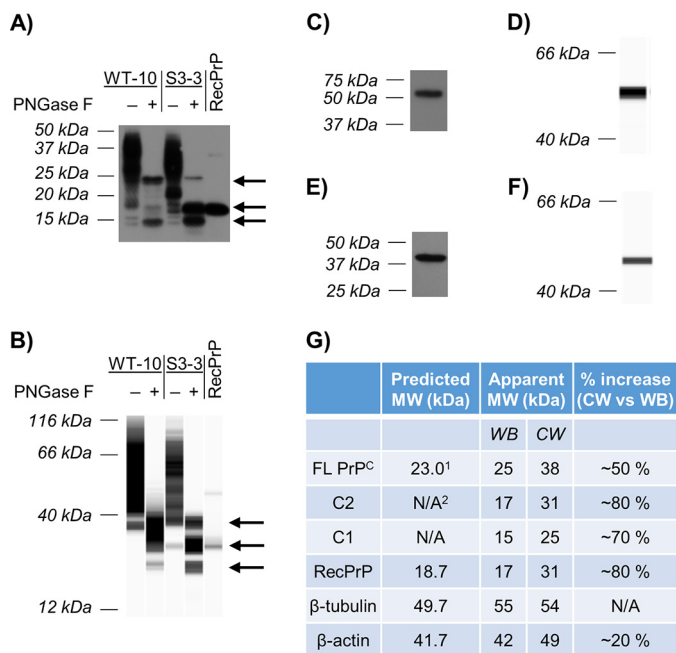


Figure 2. Comparison of protein sizing in capillary Western and traditional Western blotting assays. A and B, scan of a Western blot exposed to film (A) and a capillary Western lane view image (B) showing the apparent MWs of FL PrP^C, C2, and C1 (indicated by the arrows from top to bottom) in PNGase F-treated WT-10 and S3-3 RK13 cell lysates as well as the PrP^C immunodetection patterns in untreated lysates. The lysates were analyzed at different concentrations to adjust for variability in expression level between the cell lines, thereby ensuring optimum clarity of the immunodetection patterns. Each image also demonstrates the migration of recPrP (residues 90–231 of murine PrP^C plus a 22-amino acid N-terminal fusion tag). C–F, scans of Western blots exposed to film (C and E) and capillary Western lane view images (D and F) comparing the apparent MWs of β -tubulin (C and D) and β -actin (E and F) in RK13 cell lysates analyzed by the different systems. It should be noted that D shows lane view data from the same capillary as the left-most lane in Fig. 1C. G, increased apparent MWs of the recPrP and the deglycosylated forms of FL PrP^C, C2, and C1 in capillary Western (CW) analysis compared with traditional Western blotting (WB). Results for β -tubulin and β -actin are also summarized. Note that the apparent MWs can vary slightly; hence, the percentage increases are reported to the nearest 10%. The ExPASy Compute pI/MW tool (18) was used to calculate predicted MWs (average molecular mass setting). 1, value excludes the GPI anchor that remains after treatment with PNGase F. 2, not calculated because of uncertainty regarding the exact cleavage site (the same point applies to C1).

shifted to an apparent MW ~50% higher, and the effect was slightly greater for C1 and C2 (Fig. 2, A, B and G). Although PNGase F treatment removes N-linked glycans from FL PrP^C and its C-terminal fragments, the GPI anchor is retained. Because recPrP expressed in *Escherichia coli* is not modified by addition of a GPI anchor, we analyzed the migration of a recPrP using the different Western systems. Surprisingly, we found that the absence of a GPI anchor did not reduce the shift in apparent MW associated with capillary Western assay analysis (Fig. 2, A, B, and G). Thus, neither N-linked glycans nor the GPI anchor are responsible for the altered migration of PrP^C in the capillary Western system. Interestingly, β -tubulin in RK13 cell lysates migrated to ~54 kDa in capillary Western assays (Fig. 2D), which is similar to its apparent MW in a traditional Western blotting (Fig. 2, C and G) and to its theoretical average molecular mass of 49.7 kDa, as determined by the ExPASy Compute pI/MW tool (18). In contrast, the apparent MW of β -actin was increased when analyzed by capillary Western (Fig. 2, E and F), although the shift was more subtle than was the case for PrP^C and its cleavage fragments (Fig. 2G).

Optimizing capillary Western assay for quantification of the deglycosylated forms of full-length PrP^C and its cleavage fragments

To quantify levels of the different forms of PrP^C accurately we needed to identify the sample concentrations that fell within the linear range of the capillary Western assay. To achieve this, we tested multiplexed detection of PrP^C and β -tubulin over a range of concentrations of PNGase F-treated S3-3 RK13 cell lysates. β -Tubulin signals increased in a linear fashion from 0.1–0.6 mg/ml of total protein concentrations (Figs. 3, A and B), but higher concentrations did not result in further linear increases. In contrast, PrP^C immunostaining intensity increased in a linear manner over the entire concentration range tested; Fig. 3C displays quantification data for C2, which is the most abundant form of PrP^C in these lysates. Together, these results led us to select 0.5 mg/ml as the optimum cell lysate concentration for quantifying PrP^C expression. Example data obtained using the optimized conditions are shown in Fig. 4. To enable easy comparison with traditional Western blots, the majority of capillary Western data in this manuscript are displayed using the lane view format available in the Compass software that accompanies the Western machine. However, the lane view is generated artificially by the software from the actual data output, which takes the form of spectra of chemiluminescence signals *versus* apparent MW, this being partly akin to a densitometric plot. Fig. 4, A and B, shows example spectra from probing untreated and PNGase F-treated parental and S3-3 RK13 cell lysates for PrP^C. Parental (unmodified) RK13 cell lysates do not display detectable levels of endogenous PrP^C expression, and the almost complete absence of chemiluminescence signals from those lysates demonstrates the specificity of the Sha31 antibody. Fig. 4C illustrates that detection of PrP^C and β -tubulin can be multiplexed in PNGase F-treated WT-10 and S3-3 RK13 cell lysates without any of the bands interfering with each other.

In addition to working with cell lysates, we have analyzed PrP^C expression in brain homogenates on the capillary Western system. Fig. 5A shows the lack of PrP^C immunostaining in a *Prnp*-null homogenate compared with the spectrum obtained from homogenized TgPrP(S3.F88W)-14 brain tissue in the absence of deglycosylation; this transgenic mouse line expresses a version of S3 PrP^C known as S3.F88W, and its generation has been reported previously (13). We confirmed this result using a different anti-PrP antibody known as 12B2 (19) (Fig. 5B). PrP^C deglycosylation in brain homogenates seemed to require a higher concentration of PNGase F (15 units/ μ l) than necessary for cell lysate samples, although a 1-h incubation period was still sufficient. Fig. 5C demonstrates that these digest conditions enabled peaks corresponding to C1, C2, and FL PrP^C to be easily identified in a TgPrP(S3.F88W)-14 brain homogenate, although the peaks were less well-resolved than in cell lysate samples. The major problem, however, is that the chemiluminescence signals for the peaks shown in Fig. 5C are too high to fall within the linear range of detection of the capillary Western system; for example, the area of the FL PrP^C peak was calculated to be $\sim 7.9 \times 10^6$, far outside of the confirmed linear range (Fig. 3C). An obvious solution was to lower the signals by reducing the total protein concentration of the sample. Unfortunately, the result was that the FL PrP^C peak in particular became poorly defined, with some “shouldering” on its right-

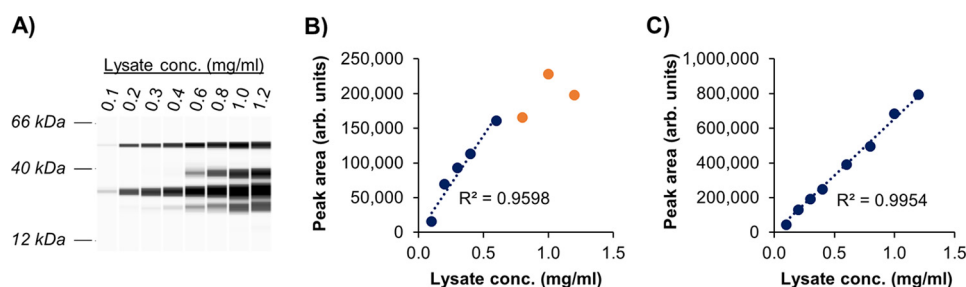


Figure 3. Assessment of linear ranges for quantification of β -tubulin and PrP^C expression levels. A, capillary Western lane view image showing multiplexed immunodetection of PrP^C and β -tubulin (top set of bands) over a range of PNGase F–treated S3-3 RK13 cell lysate concentrations. B and C, scatterplots reporting quantification of β -tubulin (B) and C2 immunodetection (C). Trend lines fitted by linear regression are shown on the plots (limited to the 0.1–0.6 mg/ml concentration range for the β -tubulin data). *arb.*, arbitrary; *conc.*, concentration.

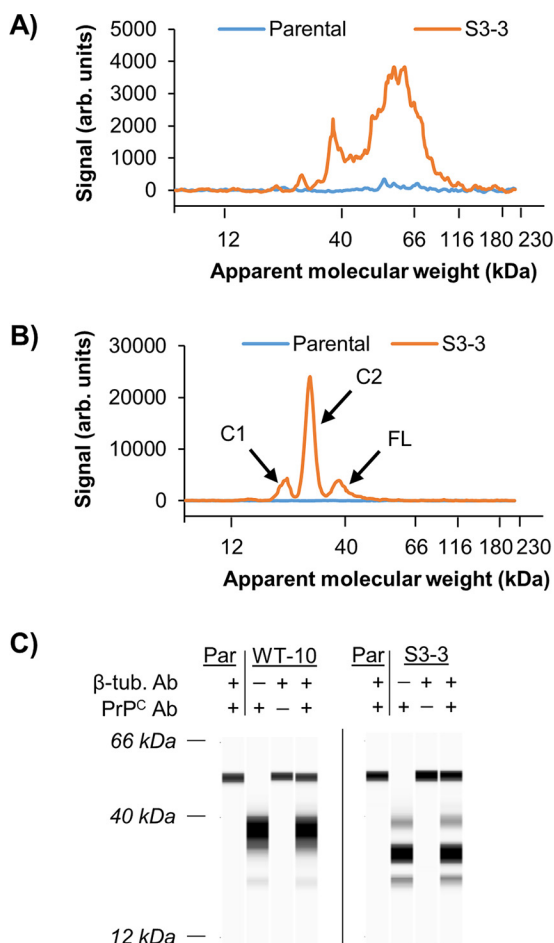


Figure 4. Detection of FL PrP^C and its cleavage fragments using optimized capillary Western assay conditions. A and B, capillary Western spectra comparing PrP^C immunodetection in untreated (A) and PNGase F–treated (B) parental and S3-3 RK13 cell lysates. C, capillary Western lane view image demonstrating multiplexed detection of PrP^C and β -tubulin in PNGase F–treated WT-10 and S3-3 RK13 cell lysates and highlighting the lack of PrP^C immunodetection in parental RK13 cell lysates. The *dividing line* indicates that different brightness/contrast settings were applied to each side of the image to adjust for differences in PrP^C expression level between the cell lines. It should be noted that the *second* and *sixth* lanes display signals from the same capillaries as the *second* and *fourth* lanes of Fig. 1B (each capillary is a separate reaction, so lanes can justifiably be arranged in any desired order). *arb.*, arbitrary; β -tub., β -tubulin; *Ab*, antibody; *par*, parental.

hand side being visible in the spectrum view (Fig. 5D). As it stands, this issue makes it challenging to quantify FL PrP^C levels accurately in brain homogenate samples.

Developing an in-plate lysis and deglycosylation regime for screening assays

Compounds that reduce overall levels of PrP^C expression or modify its processing would be potential therapeutic agents for multiple neurodegenerative disorders. The higher throughput of the capillary Western system, its potential for more accurate quantification, and its dramatically reduced sample volume requirements make microplate-based screening for such compounds more practical. However, both the cell lysis and PNGase F treatment processes represented potential bottlenecks; significant user time would be required to move each lysate individually to a microcentrifuge tube, transfer the supernatants after the clarifying spin to new tubes, and set up PNGase F digests in another set of tubes. Therefore, we developed an in-plate lysis and deglycosylation procedure. First, we modified the lysis buffer to contain DTT and a higher concentration of SDS to match the denaturing and reducing environment normally provided by adding glycoprotein denaturing buffer (New England Biolabs) to samples when preparing PNGase F digests; this change eliminated some pipetting steps and avoided the need to transfer impractically small volumes of glycoprotein denaturing buffer subsequently to each well of the microplate. Second, the clarifying spin was performed with the lysates still in the original cell culture plate, and samples of the supernatants were transferred to a fresh microplate using a multichannel pipette. Third, we omitted the 10-min incubation at 99 °C that would normally precede addition of PNGase F and its associated buffers; this step did not seem to be required for complete deglycosylation of PrP^C within 1 h.

As an initial assessment of our assay, we prepared several replicate lysates of S3-3 RK13 cells from a 96-well plate using either the in-plate or standard methods ($n = 6$ for each). Capillary Western assay analysis suggested that percentage coefficients of variation (%CV) for the loading error–corrected PrP^C chemiluminescence signals were comparable for the different methods (Fig. 6, A–D and F). When we performed a test experiment in which immunodetection of extracellular signal-related kinase 1 was quantified in 24 technical replicates of the same HeLa cell lysate, we obtained a %CV of 10.1 (Fig. 6G). In contrast, for our in-plate lysis and PNGase F treatment test, the %CV values ranged from 14.9 to 21.5 for the different forms of PrP^C (Fig. 6F). However, we considered these values to be acceptable given that the samples analyzed were separate cell lysates (*i.e.* biological, not technical replicates), albeit ones pre-

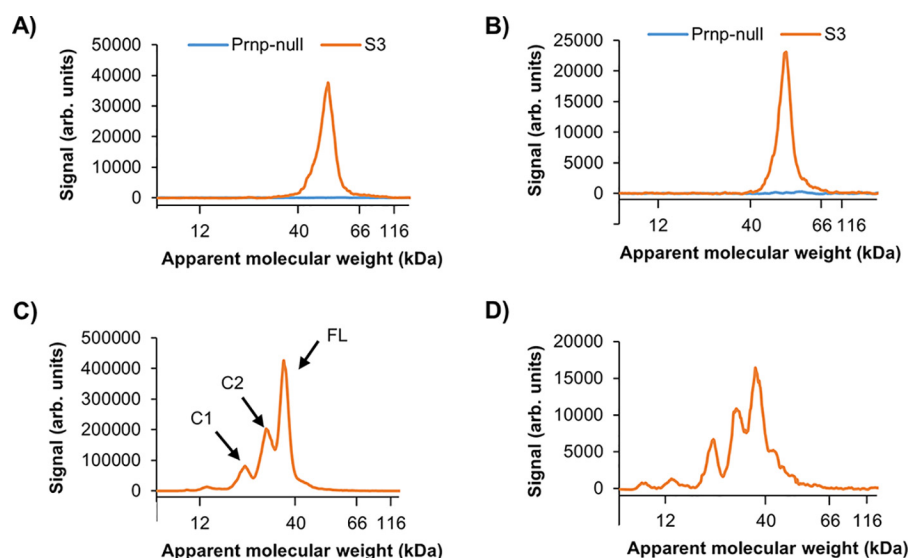


Figure 5. Capillary Western analysis of PrP^C expression in mouse brain homogenates. A and B, capillary Western spectra comparing PrP^C immunodetection with Sha31 (A) or 12B2 (B) primary antibodies in *Prnp*-null and TgPrP(S3.F88W)-14 mouse brain homogenates not treated with PNGase F. C and D, capillary Western spectra showing PrP^C immunodetection with Sha31 primary antibody in PNGase F-treated TgPrP(S3.F88W)-14 brain homogenate analyzed at 0.5 µg/µl (C) and 0.1 µg/µl (D) total protein concentrations. *arb.*, arbitrary.

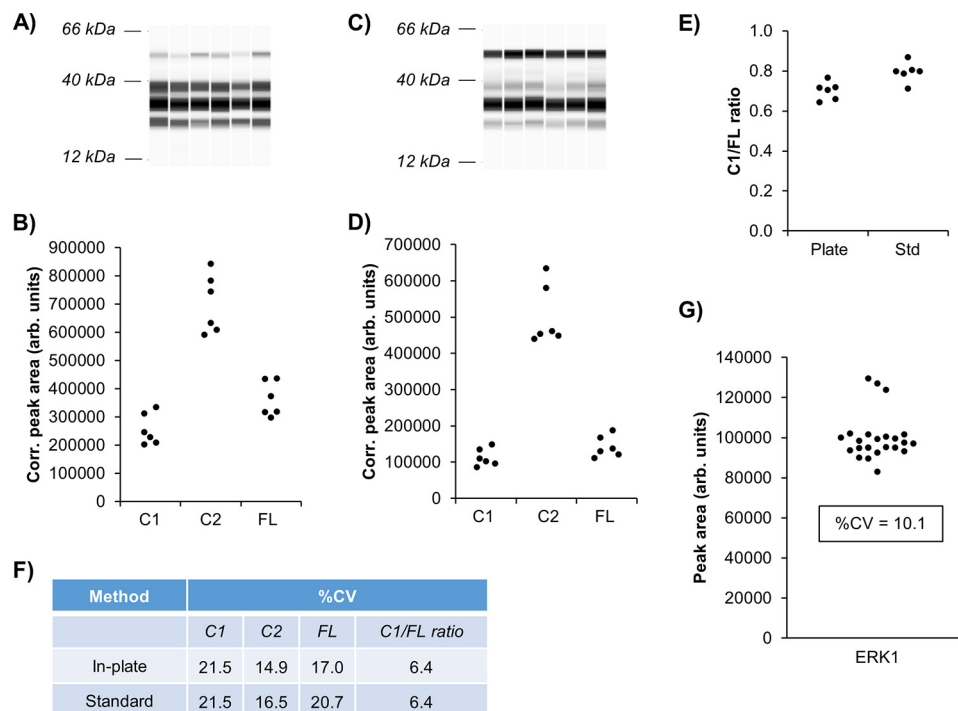


Figure 6. Comparison of in-plate lysis and deglycosylation method with standard protocols. A, capillary Western lane view image showing multiplexed immunodetection of PrP^C and β -tubulin in PNGase F-treated S3-3 RK13 cell lysates prepared by the in-plate method. Each lysate was prepared from duplicate wells of the same microplate. B, scatterplot reporting quantification of the data shown in A; β -tubulin signals were used to correct for loading error. C and D, data equivalent to A and B except that lysis and PNGase F treatment were performed using the standard methods. E, scatterplot comparing the variation in C1/FL PrP^C ratios among the replicate lysates prepared by the in-plate and standard (*std*) methods. F, table showing the %CV values for the data obtained by the different lysis and deglycosylation methods. G, scatterplot reporting quantification of immunodetection of extracellular signal-related kinase 1 (ERK1) in 24 technical replicates of a HeLa cell lysate that were analyzed in the same capillary Western assay. *corr.*, corrected; *arb.*, arbitrary.

pared as part of the same overall experiment. Moreover, the %CV for the ratio of C1 to FL PrP^C was considerably lower at just 6.4 (Fig. 6, E and F). Identifying compounds that increase this ratio will be a key target of future screening assays because such compounds ought to be neuroprotective.

In subsequent experiments, we investigated a PrP^C-binding peptide aptamer called PA8 as a potential internal control for

screening assays to identify modulators of PrP^C proteolysis. PA8 is a peptide displayed by a scaffold protein, *E. coli* thioredoxin A (trxA), and has been shown to increase PrP^C α -cleavage in the N2a cell line, resulting in higher levels of C1 (20). Exposure of WT-10 RK13 cells to 25 or 50 µg/ml PA8 aptamer for 4 days significantly increased the C1/FL PrP^C ratio compared with the trxA scaffold control treatment (Fig. 7, A and B).

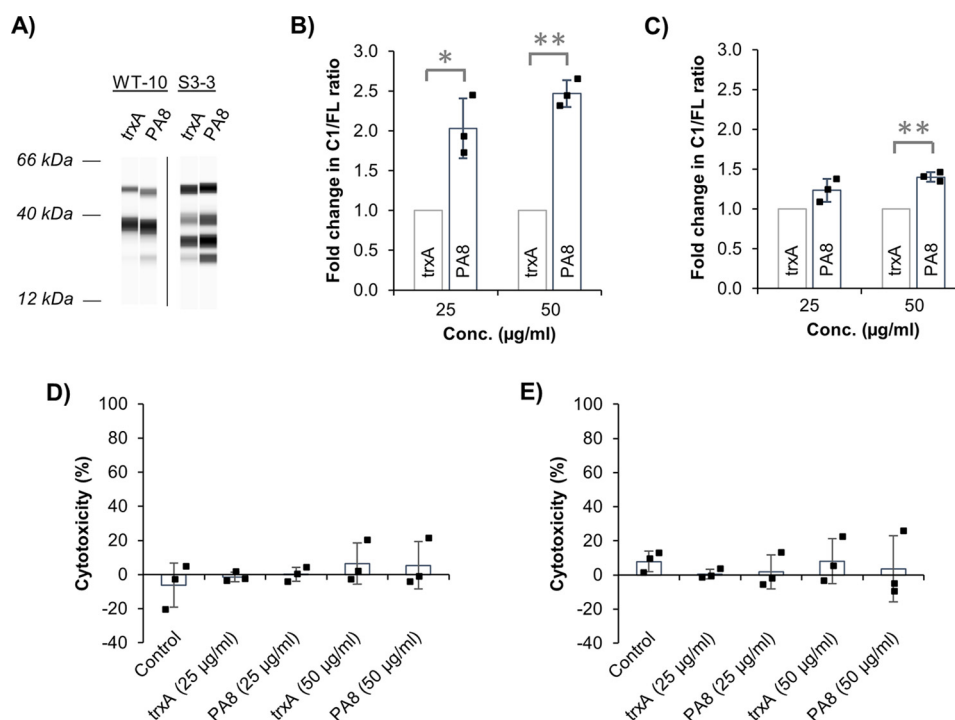


Figure 7. PA8 aptamer enhanced α -cleavage of WT and S3 PrP^C without causing significant cytotoxicity. A, representative capillary Western lane view image showing multiplexed immunodetection of PrP^C and β -tubulin in PNGase F-treated lysates of WT-10 and S3-3 RK13 cells exposed for 4 days to 50 μ g/ml of the PA8 aptamer or the trxA scaffold control (the dividing line indicates that different brightness/contrast settings were applied to each side of the image to adjust for differences in PrP^C expression level between the cell lines). B and C, bar charts showing the mean fold changes $\pm$ S.D. in the C1/FL PrP^C ratio in WT-10 (B) and S3-3 RK13 cells (C) treated with PA8 for 4 days compared with trxA-treated control cells. Statistically significant differences, as determined by one-sample *t* tests ($n = 3$ experiments), are indicated (*, $p < 0.05$; **, $p < 0.01$). The square markers represent individual data points. D and E, bar charts showing the mean percentage cytotoxicity ($\pm$ S.D., $n = 3$ experiments) in WT-10 (D) and S3-3 RK13 cell cultures (E) that were treated with trxA or PA8 for 4 days or left untreated (control). The square markers represent individual data points. Neither trxA nor PA8 treatment at either concentration resulted in significantly increased cytotoxicity, as assessed by unpaired two-sample *t* tests versus control ($p > 0.05$ in all cases). conc., concentration.

Indeed, 50 μ g/ml PA8 increased this ratio by almost 2.5-fold. Treatment with PA8 had less effect in S3-3 RK13 cells (Fig. 7, A and C), presumably because of the higher baseline level of C1 relative to FL PrP^C. However, exposure of S3-3 RK13 cells to 50 μ g/ml PA8 still resulted in a statistically significant 1.4-fold increase in the C1/FL PrP^C ratio. Importantly, neither PA8 nor trxA were notably toxic to the RK13 cell lines, as demonstrated by lactate dehydrogenase cytotoxicity assays (Fig. 7, D and E). Therefore, the PA8 aptamer will be a useful internal control for future screening assays.

Discussion

In this study, we demonstrated that PrP^C and its cleavage fragments C1 and C2 can be detected and quantified accurately using capillary Western assay analysis. We also developed methods that will enable us to use this technology for high-throughput screening experiments to identify compounds that manipulate PrP^C proteolysis or the overall expression level.

Our initial efforts to optimize PrP^C deglycosylation prior to capillary Western assay analysis demonstrated that sample degradation occurred during prolonged incubation at 37 °C with PNGase F. Furthermore, increasing the concentrations of protease inhibitors did not block this effect. One might expect the 10-min incubation at 99 °C in a denaturing and reducing buffer environment prior to addition of PNGase F to have inactivated the proteases in the samples. However, certain enzymes are resistant to SDS, such as proteinase K (21), and heat-induced

denaturation is not necessarily irreversible. Additionally, the lack of secondary and tertiary structure can make denatured proteins more accessible to proteases than native proteins (21). Therefore, higher-than-normal concentrations of protease inhibitors might be necessary to inhibit the protein degradation observed during prolonged PNGase F digests. In any case, our data suggest that researchers should be cautious about extending incubations with PNGase F beyond 1 h.

During our preliminary optimization experiments, we observed that the PrP^C immunostaining pattern in the capillary Western system differed from that obtained by traditional Western blotting. Specifically, PrP^C bands/peaks were shifted to considerably higher apparent MWs. Moreover, PNGase F treatment of RK13 cell lysates coupled with analysis of recPrP demonstrated that neither the N-linked glycans nor the GPI anchor of PrP^C caused the altered migration. Intriguingly, β -tubulin displayed a similar apparent MW when analyzed by both techniques, whereas β -actin was shifted to a slightly higher apparent MW in the capillary Western system. It seems likely that compositional differences between the proprietary capillary Western electrophoresis buffers and those used in standard SDS-PAGE are responsible for the discrepancy in protein sizing. Presumably, some feature of the amino acid composition of PrP^C caused the change in buffer system to affect migration to a greater extent than was the case for the other proteins.

Capillary Western analysis of prion protein fragments

Through further experiments, we were able to identify the range of cell lysate concentrations for which PrP^C immunostaining intensity in capillary Westerns increased in a linear fashion. We also optimized multiplexing of β -tubulin and PrP^C detection to enable correction of quantification data for loading error. One caveat associated with using β -tubulin as a loading control protein in future experiments is that we will need to ensure that its levels are not affected by the treatment in question.

In addition to cell lysate samples, we analyzed brain homogenates using the capillary Western system. Although analysis of such samples in the absence of deglycosylation is effective, we found that PNGase F treatment results in poorly defined FL PrP^C peaks that are unsuitable for quantification. At first glance, the shouldering on the FL PrP^C peaks would seem to indicate incomplete deglycosylation. However, this seems not to be the case because the shouldering was less prominent when a greater amount of the same sample was digested to enable analysis at a much higher concentration (unfortunately too high to be within the linear range of quantification). We are currently investigating potential solutions for the poor peak resolution in brain homogenate samples.

Small molecules that inhibit overall PrP^C expression levels or modulate PrP^C proteolysis may be useful therapeutic agents for neurodegenerative diseases. Compounds that enhance α -cleavage are of particular interest because the C1 fragment is reportedly a dominant-negative inhibitor of PrP^{Sc} formation (6, 7) and does not contain the putative binding sites for neurotoxic β -amyloid and α -synuclein oligomers that are present in full-length PrP^C (10–12). Ultimately, we envisage using capillary Western analyses to screen for such compounds; however, to fully exploit the higher throughput of the capillary Western system, we needed to speed up the steps required to prepare cell lysate samples for analysis. Thus, we designed and tested an in-plate method for lysing cells and removing *N*-linked glycans from PrP^C. When analyzed by capillary Western, the intra-assay variability in PrP^C signals from replicate lysates was comparable with our standard lysis and deglycosylation methods, and proof-of-principle experiments with the PA8 aptamer demonstrated that we can detect changes in PrP^C proteolysis via this approach.

The capillary Western system in our laboratory can analyze up to 24 samples simultaneously, and it will be possible for a single researcher to perform two runs on the machine per day when using the in-plate lysis and deglycosylation procedure. Overall, we estimate that this approach will more than halve the person time required to screen a compound library compared with using our standard lysis, deglycosylation, and Western blot methods. Furthermore, although the procedure reported in this manuscript still includes several manual pipetting steps, a liquid-handling robot could potentially automate most if not all of the steps, including transferring deglycosylated lysates to 0.2-ml strip tubes for the sample preparation steps that precede capillary Western assay analysis.

As well as being used for the screening assays already mentioned, the in-plate lysis and deglycosylation method could be applied for other purposes, such as RNAi screens to identify the proteases that process FL PrP^C. As it stands, the identity of the

protease responsible for α -cleavage is up for debate (reviewed in Ref. 22). Additionally, although it has been reported initially that β -cleavage is protease-independent, driven instead by reactive oxygen species in a copper-dependent manner (4), cleavage of recPrP by ADAM8, a member of the “a disintegrin and metalloproteinase” enzyme family, has been demonstrated *in vitro* (3). Recent results from our laboratory also hint at an enzymatic explanation for β -cleavage (13).

As far as we are aware, this is the first study to report analysis of PrP^C and its cleavage fragments using novel capillary Western technology. We believe that this approach can deliver insights into PrP^C biochemistry and accelerate efforts to identify potential therapeutics for prion diseases and other neurodegenerative disorders.

Experimental procedures

Cell culture and treatments

Rabbit kidney epithelial (RK13) cells (originally purchased from the American Type Culture Collection) were routinely cultured in Dulbecco's modified Eagle's medium (containing 1 g/liter D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate) supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin (all reagents were purchased from Fisher Scientific). This medium formulation was used for all experiments except for cytotoxicity assays (the fetal bovine serum concentration was reduced to 5%). Cells were passaged every 3–5 days and grown at 37 °C in 5% CO₂ and 95% humidity.

WT-10 and S3-3 stable cell lines were generated from the parental RK13 cell line according to methods published previously (13). WT-10 stably expresses WT murine PrP^C, and S3-3 stably expresses PrP^C with a mutated octapeptide repeat region that is referred to as S3 PrP^C (13).

Cells were transferred to Corning Falcon 96-well tissue culture plates for experiments and were allowed to recover for 24 h before lysis or further treatment. Cells were exposed to the PA8 aptamer or the trxA control for 4 days. The culture medium and aptamers were refreshed halfway through the incubation period. The generation and characterization of the PA8 aptamer have been reported previously (20, 23).

Cytotoxicity assays

Within each experiment, quadruplicate wells of cells were used for each experimental condition. 10 $\times$ lysis solution from the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, G1780) was added to two of the wells for each condition (condition-specific high controls). After a 45-min incubation, 50- μ l samples of conditioned culture medium were transferred from each cell-containing well to a 96-well microplate (Greiner, 655201) and assayed for lactate dehydrogenase activity according to the Promega kit instructions. Absorbance at 490 nm (reference wavelength, 650 nm) was measured using a μ Quant microplate spectrophotometer (Bio-Tek). The mean signal from culture medium—only wells was subtracted from the signals from the cell-containing wells. For each experimental condition, 100% cytotoxicity was set to the mean background-corrected signal from the condition-specific high control wells. This modified method for calculating percentage cytotoxicity

controls for any effect of the experimental treatment on cell proliferation, which would otherwise result in artifactually low or high cytotoxicity values (24).

Cell lysis

Cell monolayers were washed twice with ice-cold PBS prior to lysis. For the standard method, cells were incubated on ice for 15 min in radioimmune precipitation assay lysis buffer (50 mM Tris, 150 mM NaCl (pH 7.4), 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS) supplemented with complete protease inhibitor mixture (Roche). Lysates were scraped into tubes and clarified by centrifugation at $15,000 \times g$ for 10 min at 4 °C. Total protein concentrations of the supernatants were determined by bicinchoninic acid assay (Pierce).

For the in-plate method, the aforementioned radioimmune precipitation assay buffer was modified by addition of 26 mM DTT and by changing the SDS concentration to 0.32% (w/v). Instead of scraping lysates into tubes, the tissue culture plates were centrifuged at $1000 \times g$ for 10 min at 4 °C to pellet cell debris. Supernatants were transferred to fresh 96-well microplates (Greiner, 655201) for PNGase F treatment.

PNGase F treatment

All reagents were purchased from New England Biolabs unless stated otherwise. For the standard method, cell lysates or brain homogenates were denatured by incubation at 99 °C for 10 min in the presence of glycoprotein denaturing buffer. NP-40 was added to the reaction to a final concentration of 1.25% (v/v) in addition to GlycoBuffer 2 and either 5 units/ μ l (for cell lysates) or 15 units/ μ l PNGase F (for brain homogenates). Protein samples were deglycosylated by incubation at 37 °C in an Eppendorf thermocycler for varying time periods. On occasions when additional protease inhibitors were required, complete protease inhibitor mixture (Roche), at the manufacturer's recommended concentration, was added to the reaction prior to incubation at 37 °C. Total protein concentrations in the digests varied according to the requirements of the individual experiment but did not exceed 1.5 mg/ml. For the in-plate method, the procedure was the same, except that the 99 °C incubation step was omitted, and the digest phase was performed in a 37 °C incubator.

Recombinant prion protein production

The recPrP used in this study consisted of residues 90–231 of murine PrP^C fused to a 22-amino acid N-terminal tag (MGSSHHHHHSSGLVPRG SHML). The expression and purification of this protein has been reported previously (25).

Transgenic mouse lines and brain homogenization

All animal procedures and husbandry were performed in accordance with Canadian Council on Animal Care guidelines and local institutional protocols (AUP00000356). The generation of FVB transgenic mice expressing the S3.F88W mutant form of PrP^C has been reported previously (13); the line used here is referred to as TgPrP(S3.F88W)-14. The control line of *Prnp*-null mice was also in the FVB background and derives from the original *Zrch1* knockout line (26).

Mice were sacrificed by cervical dislocation. Tissue was immediately extracted, frozen on dry ice, and kept at –80 °C until use. Hemisected brain samples were homogenized manually at 20% (w/v) concentration in PBS containing 2% (v/v) Sarkosyl and complete protease inhibitor mixture (Roche). Total protein concentrations were determined by bicinchoninic acid assay (Pierce).

Capillary Western assays

Reagents and equipment were purchased from ProteinSimple unless stated otherwise. Cell lysates or brain homogenates were diluted using a proprietary sample buffer when required. 5X Fluorescent Master Mix was added to each sample to provide a denaturing and reducing environment (final concentrations of 1% (v/v) SDS and 40 mM DTT). Samples were incubated at 95 °C for 5 min. Four microliters of each sample were loaded into the top-row wells of plates preloaded with proprietary electrophoresis buffers designed to separate proteins of 12–230 kDa. Each plate could accommodate 24 samples alongside a biotinylated Simple Western size standard. Subsequent rows of the plate were filled with blocking buffer (antibody diluent 2), primary and secondary antibody solutions, chemiluminescence reagents, and wash buffer according to the manufacturer's instructions. Primary antibodies were Sha31 anti-PrP (Spi-Bio Inc., A03213, raised in mice, diluted 1:10,000), 12B2 anti-PrP (see Ref. 19, raised in mice, diluted 1:500), anti- β -actin (Sigma, A5441, raised in mice, diluted 1:1250), anti- β -tubulin (Novus Biologicals, NB600-936, raised in rabbits, diluted 1:200 or 1:400), and anti-extracellular signal-related kinase 1 (ProteinSimple, 042-486, raised in rabbits, used undiluted). Primary antibodies were diluted in antibody diluent 2. Secondary antibodies were anti-mouse secondary HRP conjugate and anti-rabbit secondary HRP conjugate. The HeLa cell lysate was provided by ProteinSimple for testing purposes (042-488).

Plates and capillaries were loaded into a Wes machine, and assays were performed using the standard 12- to 230-kDa separation range protocol that was introduced upon release of version 3.1.7 of the accompanying Compass software, except that the separation time was changed to 30 min from the default 25 min. Compass reports data as spectra of chemiluminescence signals *versus* apparent MW. The software determines apparent MWs by mapping ladder peaks to capillary positions and using the signals from fluorescently labeled protein standards of known MW (present in the 5X Master Mix) to adjust for any differences in migration among capillaries. Peak area calculations were performed by the Compass software using the default Gaussian method unless the fitted peaks mapped poorly to the actual peaks in the spectrum; in such cases, the “dropped lines” method was used for quantification. Compass was also used to generate artificial lane view images from the spectra. Visual adjustment to lane view images was performed solely using the brightness/contrast slider within the software.

Western blotting

Reduced and denatured cell lysate samples were loaded into hand-cast 14% Tris/glycine gels and were separated by SDS-PAGE using a Bio-Rad Mini-PROTEAN Tetra cell. Size-separated proteins were transferred to polyvinylidene fluoride

membranes using a Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell. Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 for 1 h at room temperature before being incubated overnight at 4 °C with primary antibodies diluted in blocking buffer (anti- β -actin and anti- β -tubulin used at 1:1250 and 1:2500 dilutions, respectively). When Sha31 anti-PrP primary antibody was used, the initial blocking step was omitted, and membranes were incubated immediately with Sha31 diluted 1:30,000 in TBS/0.5% Tween 20. After four washes with TBS/0.1% Tween 20, membranes were subsequently incubated for 1 h at room temperature with goat anti-mouse IgG–HRP conjugate (Bio-Rad, 170-6516) or goat anti-rabbit IgG–HRP conjugate (Bio-Rad, 172-1019) diluted 1:10,000 in blocking buffer. After four additional washes, staining was visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, PI32106). Membranes were exposed to X-ray films that were subsequently scanned using a Fluor Chem E Imager (ProteinSimple). Adjustment of brightness/contrast was performed in ImageJ.

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