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1 **Counting of single prion particles bound to a capture-antibody**  
2 **surface (surface-FIDA)**

3  
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17  
18 **Abstract**

19 Hitherto accredited prion tests use the PK resistance of PrP<sup>Sc</sup>, the pathogenic isoform  
20 of the prion protein, as a marker for the disease. Because of variations in the amount of  
21 disease-related aggregated PrP, which is not PK-resistant, these prion tests offer only limited  
22 sensitivity. Therefore, a prion detection method that does not rely on PK digestion would  
23 allow for the detection of both PK-resistant as well as PK-sensitive PrP<sup>Sc</sup>. Furthermore, single  
24 particle counting is more sensitive than methods measuring an integrated signal. Our new test  
25 system is based on dual-colour fluorescence correlation spectroscopy (FCS). This method

26 quantifies the number of protein aggregates that have been simultaneously labelled with two  
27 different antibodies using dual-colour fluorescence intensity distribution analysis (2D-FIDA).  
28 This only counts PrP aggregates, and not PrP monomers. To increase the sensitivity, PrP<sup>Sc</sup>  
29 was concentrated in a two-dimensional space by immobilizing it so that the antibodies could  
30 be captured on the surface of the slide (surface-FIDA). When the surface was systematically  
31 scanned, even single prion particles were detected. Using this new technique, the sensitivity to  
32 identify samples from scrapie-infected hamster as well as BSE-infected cattle can be  
33 dramatically increased in comparison with identification using FIDA in solution.

34

35 **Keywords:** Prion; proteinase K-free diagnosis; TSE diagnosis; single particle detection;  
36 surface-FIDA; BSE, scrapie, CSF

37

## 38 1. Introduction

39 Prions are the causing agent of transmissible spongiform encephalopathies (TSEs)  
40 such as Creutzfeldt-Jakob disease (CJD) in man, bovine spongiform encephalopathy (BSE) in  
41 cattle and scrapie in sheep. These diseases are characterised by an abnormally folded form of  
42 the host-encoded prion protein (PrP) (Prusiner, 1998). The cellular, i.e. non-pathological  
43 isoform of PrP (PrP<sup>C</sup>), is present in most tissues and is most abundant in the central nervous  
44 system. Following infection, PrP<sup>C</sup> undergoes a conformational change during a post-  
45 translational process, leading to altered physicochemical properties such as aggregation,  
46 insolubility and  $\beta$ -sheet rich secondary structure. This pathological isoform is designated  
47 human PrP<sup>Sc</sup>, bovine PrP<sup>Sc</sup> or hamster PrP<sup>Sc</sup>.

48 Through digestion with proteinase K (PK), PrP<sup>Sc</sup> is N-terminally truncated at amino  
49 acids 89/90, the C-terminal part is resistant against further PK digestion but still fully  
50 infectious (resPrP). The PK resistance is accounted for as a marker for the pathological

51 isoform of PrP, and is the basis for most commercially-available TSE tests. However, a  
52 portion of the disease-related PrP<sup>Sc</sup> is not PK resistant. These properties have been described  
53 quantitatively for several hamster prion strains (Safar et al., 1998) and for human PrP<sup>Sc</sup> (Safar  
54 et al., 2005). In summary, the disease-related PrP<sup>Sc</sup> is always aggregated, but not all PrP<sup>Sc</sup> is  
55 PK resistant. Consequently, it is likely that aggregated PrP<sup>Sc</sup> is a more sensitive TSE marker  
56 than PK-resistant PrP.

57       Following the emergence of BSE (Wilesmith et al., 1991) as well as "new variant"  
58 CJD (vCJD) along with the strong evidence that they were connected (Bruce et al., 1997; Hill  
59 et al., 1997, Lasmezas et al., 1996), BSE diagnosis became a matter of great scientific and  
60 public interest. Scrapie in sheep was also the focus of diagnosis, due to the potential danger of  
61 BSE transmission to sheep (Jeffrey et al., 2001).

62       As mentioned above, most diagnostic methods currently available are based on the  
63 PK-resistant C-terminal part of PrP detected by ELISA or Western immunoblotting (Grassi et  
64 al., 2000; Oesch et al., 2000; Ingrosso et al., 2002). The conformation-dependent  
65 immunoassay (CDI: Safar et al., 1998; 2002; 2005) does not depend totally on PK resistance  
66 but contains a PK-digestion step in its most sensitive version. These techniques are applied  
67 post-mortem, and the infection can only be detected at the onset of clinical signs or, at best,  
68 some months earlier. A test which could be conducted on living animals or a test on  
69 slaughtered animals very early after the infection would be desirable. Therefore, the detection  
70 of very small amounts of PrP<sup>Sc</sup> aggregates, ideally, even single aggregates, is necessary.

71       In an earlier study we described a method of detecting bovine PrP<sup>Sc</sup> and hamster PrP<sup>Sc</sup>  
72 aggregates prepared from brain samples without PK digestion by fluorescence correlation  
73 spectroscopy (FCS) (Birkmann et al., 2006). In that method, originally developed by Eigen  
74 and Rigler (1994), a laser beam is focused on a sample volume with a diameter of less than a  
75 micrometer, and the excited fluorescence light is imaged confocally onto the detector. The

76 fluctuations of the fluorescence intensity within that femtolitre volume can be evaluated. If  
77 the particle number is low, the fluctuations from fluorescent PrP mono- or oligomers can be  
78 differentiated from large PrP aggregates. FCS was applied in a dual-colour mode so that only  
79 coincidental signals of both fluorescent markers within the confocal volume were counted as  
80 specific events by cross-correlation evaluation. The system has already been applied in the  
81 monocolour mode to the detection of the A $\beta$ -peptide aggregates in the cerebrospinal fluid of  
82 Alzheimer's disease patients (Pitschke et al., 1998), in the dual colour mode for prion  
83 particles in the cerebrospinal fluid of CJD patients (Bieschke et al., 2000) In the latter case,  
84 the diagnostic sensitivity was only 20%. PrP aggregates purified, however, without any PK-  
85 digestion step, of SHa scrapie and BSE brain samples could be detected by FCS (Birkmann et  
86 al., 2006). In these studies, the detection of the particles was carried out in solution, and only  
87 a portion of all particles present in the sample volume could be detected.

88 One problem in the set-up of the former method was that aggregates show either very  
89 slow diffusion or no detectable movement at all within the time of measurement. In the worst  
90 case, the aggregates even sink to the bottom of the cuvette and vanish from the detectable  
91 volume. In the case of very low concentrations of the aggregates in particular, a second  
92 problem is that only a small part of the sample could be analysed. This was due to the very  
93 small detection volume of the FCS in comparison with the sample volume. In combination,  
94 these two problems lead to the large scattering of the measured number of particles and  
95 limited sensitivity. In the present study we present a new experimental set-up called surface-  
96 FIDA which avoids these problems. It was possible to raise the sensitivity to the extent that  
97 individual prion particles could be counted in the cerebrospinal fluid of BSE-infected cattle.

98

## 99 2. Materials and Methods

### 100 2.1. Proteinase K assays

101 Proteinase K (PK) was added to brain homogenate samples to the final concentration  
102 of 5 µg/ml. The sample was agitated for 1 hour at 37°C. Then the digestion was stopped by  
103 adding one volume loading buffer containing 5 mM PMSF and boiling for 10 min.

104

### 105 2.2. SDS/PAGE and Western Blotting

106 SDS/PAGE (12%) was carried out according to Laemmli's protocol (1970). Proteins  
107 were transferred onto PVDF membranes (Millipore, Schwalbach, Germany) (Towbin et al.,  
108 1979). The membrane was blocked with 5% fat-free dried milk in TBST buffer (10 mM Tris-  
109 HCl [pH8.0], 150mM NaCl, 0.05% Tween 20) for 1 hour and subsequently incubated with  
110 anti-PrP monoclonal antibody Saf32 (1:10000 in TBST) (Kascsak et al., 1987). The detection  
111 was carried out with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch,  
112 Newmarket, Suffolk, UK) and enhanced chemiluminescence system (Amersham Biosciences,  
113 Buckingham, GB).

114

### 115 2.3. Purification of PrP<sup>Sc</sup> and bovine PrP<sup>Sc</sup> without PK treatment

116 The purification of PrP<sup>Sc</sup> was carried out as described earlier (Birkmann et al., 2006).  
117 Bovine PrP<sup>Sc</sup> was isolated to high purity using a modified protocol (Fig. 2A) of the NaPTA-  
118 precipitation (Safar, 2002). BSE-infected cattle were killed in the terminal stage of disease,  
119 their brains were immediately frozen and stored at -70°C. A part of the *medulla oblongata*  
120 was homogenized on ice by three 30-second strokes with a PowerGen homogeniser (Fisher  
121 Scientific, Schwerte, Germany) in PBS pH 7.4 containing a protease inhibitor mix (complete  
122 mini, EDTA-free, Roche, Mannheim, Germany). The resulting homogenates 10% (w/v) were

123 mixed 1:1 with 8.4 % sarkosyl in PBS pH 7.4 and mixed three times for 30 seconds with a  
124 homogeniser at medium speed. The resulting homogenates were spun down for 1 min at 5 000  
125 x g and the supernatant was either used immediately or stored at -70°C. The negative control  
126 animals were treated the same way. Benzonase (Merck, Darmstadt, Germany) was added to a  
127 final concentration of 50 units/ml to brain homogenate (5% w/v) containing 4.2% sarkosyl  
128 and agitated for 45 min at 37°C. Then the sample was mixed with stock solution containing  
129 4% sodium phosphotungstate (NaPTA) and 170 mM MgCl<sub>2</sub> pH 7.4 to a final concentration of  
130 0.25% NaPTA / 10.6 mM MgCl<sub>2</sub> and agitated for four hours at 37°C. After a centrifugation  
131 step of 14 000 x g (EBA 12, Hettich Zentrifugen, Tuttlingen, Germany) for 30 min the  
132 resulting pellet was washed with 200 µl of PBS 200 mM EDTA pH 8 for 30 min at 37°C and  
133 centrifuged as before. The same washing step was repeated with PBS 0.1% sarkosyl. Finally  
134 the pellet was re-suspended in PBS by brief sonication (Bandelin sonopuls, Bandelin  
135 electronic, Berlin, Germany).

136

#### 137 *2.4. Fluorescence labelling of antibodies*

138 R1 Antibodies were kindly provided by S.B. Prusiner, UCSF, USA (Williamson et al.,  
139 1998). Antibodies 12F10 and Saf32 were obtained from SpiBio (Massy Cedex, France);  
140 antibody D18 was obtained from InPro (San Francisco, USA). Antibodies were labelled in  
141 free amino groups via reactive succinimidyl ester groups of Alexa-633 and Alexa-488  
142 (Molecular Probes, Oregon, USA). For labelling, approximately 50 µg antibodies were  
143 incubated with 5 µg dye in carbonate buffer, pH 8.4, in a total volume of 100 µl for 1 hour.  
144 Conjugates were separated from free dye by gel filtration via NAP5-column (Pharmacia) with  
145 10 mM TBS, pH 7.2 and 0.2 M NaCl as elution buffer. The labelled antibodies were stored in  
146 the dark at 4°C.

147



148 *2.5. Fluorescence correlation spectroscopy*

149 In fluorescence correlation spectroscopy (FCS) the fluorescence intensity is recorded  
150 in a very small volume, i.e. in the femtolitre range. Measurements were performed with the  
151 instrument FCS Olympus IX 50 (Evotec OAI, Hamburg, Germany) with a beam scanner unit  
152 in dual-colour mode with an Argon ion laser (excitation wavelength 488/514 nm) and a  
153 helium-neon laser (excitation wavelength 633 nm). Each sample was measured 3 times for  
154 210 seconds. The beam scanner unit allows the scanning of the sample for aggregates. In  
155 practice the detection volume is moved through the sample in horizontal and vertical  
156 dimensions. The beam scanner was used by moving 1 mm in one direction a rectangular  
157 deviation of 100  $\mu\text{m}$  with a frequency of 50 Hz and an integration time of 50  $\mu\text{s}$ . These  
158 settings were applied seven times, site by site, in each sample. The statistical evaluation was  
159 carried out as described in Birkmann et al. 2006.

160

161 **3. Results**

162 *3.1. The concept of surface-FIDA*

163 The detection of prion aggregates in suspension using the dual-colour fluorescence  
164 intensity distribution analysis (2D-FIDA) (Birkmann et al., 2006) was usually done in a small  
165 volume taken from a much larger sample volume by moving the laser detection focus through  
166 a cuvette. Diffusion of the particles and scanning of the volume were superimposed so that it  
167 was difficult to account quantitatively for all particles in the sample.

168 The search for a solution to this problem led to the development of the surface-FIDA  
169 assay. With the surface-FIDA assay, the prion aggregates were immobilized on a surface  
170 similar to that of an ELISA experiment in order to reduce the sample volume from a three-  
171 dimensional volume to a two-dimensional surface. In contrast to the ELISA method, no  
172 integrated signal was measured. Instead, single particles were counted by scanning.

173 Superimposition of diffusion and scanning is completely avoided and scanning can be adapted  
174 to the whole surface where all particles are fixed. A schematic presentation of the surface-  
175 FIDA setup is shown in figure 1.

176

177 *3.2. Purification of full-length bovine PrP<sup>Sc</sup> from cattle brain homogenate without PK*  
178 *treatment*

179 The full-length PrP<sup>Sc</sup> was purified from hamster brain homogenate without PK  
180 treatment following a modified protocol based on PTA precipitation (Safar et al., 1998) as  
181 described earlier (Birkmann et al., 2006). For purification of full-length bovine PrP<sup>Sc</sup> from  
182 brain homogenate without PK treatment, the protocol shown in Figure 2A was used. Two  
183 washing steps were added to the original PTA precipitation protocol with BSE material (Safar  
184 et al., 2002) and nucleases were used to enhance the purity of the samples (see Materials and  
185 Methods). The main difference was that the purification completely avoided any PK  
186 treatment.

187 The analysis of the single steps of the precipitation is shown in Figures 2 B and C. One  
188 of the BSE samples contains a very low amount of PK-resistant PrP as one can see in the PK-  
189 treated starting material, where only a light shadow of the resistant bovine PrP<sup>Sc</sup> can be seen  
190 at 30 kDa (Fig 2B, bh +). No PrP<sup>C</sup> could be detected in the resulting pellet of the negative  
191 control (Fig 2C, p -). Therefore, it can be concluded that the PrP detected in the BSE pellet  
192 fraction consists only of bovine PrP<sup>Sc</sup>, which means a combination of PK-sensitive but  
193 aggregated bovine PrP<sup>Sc</sup> and of PK-resistant bovine PrP<sup>Sc</sup> (Fig 2B, p -).

194

195 *3.3. Immobilisation and detection of prion aggregates on a glass surface with the surface-*  
196 *FIDA set-up*

197 To bind the capture antibody to the glass surface of the assay chip, the chip surface  
198 was activated with poly-D-lysine and the capture antibody bound by adhesion. For this  
199 purpose, the glass surface of the assay chips was singed and 20  $\mu\text{l}$  of poly-D-lysine (10 $\mu\text{g}/\mu\text{l}$ )  
200 were incubated in the wells for one hour at 37°C. For scrapie, prions prepared from brain  
201 homogenate antibody R1 (Williamson et al., 1998), in the case of BSE SAF32 (Krasemann et  
202 al., 1999) or D18 (Peretz et al., 2001), were used as capture, with 1 $\mu\text{g}$  per well being used. To  
203 bind the PrP particles, 20  $\mu\text{l}$  of the suspension was agitated in the wells for two hours,  
204 unspecific binding sites were blocked by incubation with 5% BSA before applying the  
205 fluorescence-labelled detection antibodies (0.1 $\mu\text{g}/\text{ml}$ ). In the case of scrapie we used R1 and  
206 D13 and for BSE SAF32 and 12F10. After three washing steps with TBST, we executed the  
207 FIDA measurements in the special surface-FIDA set-up.

208 To arrange the FCS focus in a defined distance on top of the surface of the assay chip,  
209 a piezoelement was installed in order to control the focus of the fluorescence correlation  
210 spectroscopy (Olympus IX 50, Evotec OAI, Hamburg, Germany) with an accuracy of 100 nm.  
211 Because we did not know in advance which height is most suited for the detection of the  
212 labelled aggregates, measurements were taken out at different heights above the glass surface.

213

214 *3.4. Analysis of PrP aggregates from scrapie-infected hamsters and from BSE-infected cattle*  
215 *by surface-FIDA*

216 Prion aggregates purified from scrapie-infected hamster brain homogenate were  
217 immobilized on a glass surface as described above. Control samples from non-infected  
218 hamster brains were treated the same way. Measurements were carried out at heights of 0 to  
219 20  $\mu\text{m}$  above the glass surface in order to determine the best height (Fig. 3A, B).

220 At a height of 0  $\mu\text{m}$ , high background signals were detected in the negative control,  
221 which were probably caused by unspecifically bound fluorescence or by high scattering from  
222 the surface. At a height of 5  $\mu\text{m}$ , only background signals of low intensity were detected,  
223 whereas in the scrapie-positive sample many fluorescence peaks occurred. At a height of  
224 between 10  $\mu\text{m}$  and 20  $\mu\text{m}$ , peaks could be detected in the scrapie sample in contrast to the  
225 negative control, but the number of peaks decreased with increasing height from the surface.

226 After the analysis of one scrapie sample and one negative control, samples from four  
227 different scrapie-infected animals were examined and the samples treated identically. Samples  
228 from four non-infected animals were used as negative controls. In Figure 4 the mean number  
229 of peaks is shown as histogram. The mean values are calculated as described in Materials and  
230 Methods. We can clearly distinguish the scrapie samples from the control group in the heights  
231 from 10  $\mu\text{m}$  to 20  $\mu\text{m}$  from the surface. Best results were achieved at a height of 10  $\mu\text{m}$ , as  
232 indicated by the largest number of peaks. The third scrapie sample at 10  $\mu\text{m}$  showed a  
233 remarkably high number of peaks in comparison to the other three scrapie samples measured  
234 at the same height. We have no explanation for the difference in the peak number distribution.  
235 It seems likely that the size distribution of the prion particles may have been very different in  
236 these samples.

237 BSE prion aggregates purified from *medulla oblongata* of BSE-infected cattle were  
238 also examined and negative controls treated the same way (Fig. 5). The height above the glass  
239 surface varied between 10  $\mu\text{m}$  to 20  $\mu\text{m}$ . Infected animals and the control group could be  
240 clearly distinguished. At a height of 20  $\mu\text{m}$  in the third BSE sample, the number of peaks  
241 decreased notably, so that heights from 10 to 15  $\mu\text{m}$  turned out to be the most effective.

242

243 *3.5. Test for BSE infection in the cerebrospinal fluid (CSF) of cattle*

244           The applicability of surface-FIDA to cattle CSF was tested with samples consisting of  
245 20  $\mu$ l of crude CSF with no further treatment. Because we initially only had two different  
246 samples available, we tested these two CSF samples from BSE-infected cattle in the clinical  
247 state and two negative control samples from healthy cattle with different combinations of  
248 capture and detection antibodies (Fig. 6). We were able to distinguish the BSE samples from  
249 the control samples with all antibody combinations applied. The combination of D18 as  
250 capture and SAF32 with 12F10 as detection antibodies seemed to be the best. But as we had  
251 only analysed two samples we were not able to ascertain whether surface-FIDA is generally  
252 adaptable for detecting BSE in CSF. Therefore we applied six other samples and controls to  
253 the surface-FIDA assay with the D18 as capture and SAF32 and 12F10 as probes (Fig. 7).  
254 Three of the six BSE-infected cattle could be clearly distinguished from the control group.

255

256

#### 256 4. Discussion

257 Several diagnostic methods are routinely applied to detect TSE-afflicted animals. In  
258 the present study we used a characteristic feature of spongiform encephalopathies which is  
259 not used in the routine tests mentioned above. This feature is the insolubility of prions or, in  
260 molecular terms, the aggregated state of bovine PrP<sup>Sc</sup> or hamster PrP<sup>Sc</sup>, whether it is PK-  
261 resistant or not.

262 In an earlier publication (Birkmann et al., 2006) we described the basic method of  
263 detecting aggregated PrP from scrapie-infected hamsters and BSE-infected cattle using FCS  
264 to count single fluorescent particles. Fluorescence-labelled antibodies were bound to  
265 aggregated PrP thus making the aggregates highly fluorescent. When the PrP particles cross  
266 the laser beam in suspension, they are detected as fluorescence bursts. This method enabled  
267 the distinction of BSE-infected cattle, as well as scrapie-infected hamsters, from control  
268 groups using brain homogenate. But these results also showed clearly that the standard  
269 variation resulting from measuring the same sample ten times was very high. The problems  
270 associated with this set-up were described in the Results section. A new set up was developed  
271 which results in a more accurate determination of prion aggregates in the whole sample, but  
272 which is still able to detect single particles. This new set-up was named surface-FIDA,  
273 because the prion particles were immobilised on the surface of an assay chip. The  
274 immobilised PrP aggregates were systematically scanned and counted.

275 The surface-FIDA assay has several important advantages compared to the earlier FCS  
276 studies:

- 277 - The PrP aggregates are concentrated through the immobilisation from a three-  
278 dimensional solution to a two-dimensional surface. This set-up should enable the  
279 detection of all prion particles present in a sample.

- 280 - The assay no longer depends on the superimposition of diffusion and scanning. In  
281 contrast, the FCS method is carried out in solution, the detection of the particles either  
282 by diffusion of the particles or moving of the laser beam does not require two distinct  
283 steps.
- 284 - Surface-FIDA is a combination of four steps with independent specificity.  
285 These are:
- 286 (i) PrP-specific capture on the surface;  
287 (ii) and (iii) labelling by two independent PrP-specific antibodies, which are detected  
288 as a cross-labelling signal;  
289 (iv) detection of large prion specific PrP-aggregates by FIDA.

290 In order to prove the applicability of surface-FIDA, we used PrP<sup>Sc</sup> purified from brain  
291 homogenate of scrapie-infected hamsters in the clinical state prepared without a PK-digestion  
292 step. We were clearly able to distinguish between infected animals and a control group in a  
293 test series of four infected and four healthy animals. The standard variations of the results  
294 were much smaller when comparing the 2D-FIDA experiments with those done with the same  
295 material in suspension (Birkmann et al., 2006).

296 After proving the principle, the same test series was carried out with four BSE-  
297 infected cattle and four negative control samples. A clear differentiation between infected and  
298 control animals was achieved. Also in that series, the standard variations were much lower in  
299 comparison with earlier experiments with the same samples in solution.

300 Using the surface-FIDA method, we also tested CSF samples. In the literature it was  
301 reported that in CSF of CJD patients, no PK-resistant human PrP<sup>Sc</sup> was found (Wong et al.,  
302 2001). However, the transmission rate with CSF from patients with CJD to primates was 15%  
303 and a SIFT analysis detected PrP particles in 21% of the CSF samples of CJD patients  
304 (Bieschke et al., 2000). No infectivity could be detected by inoculation in transgenic mice in

305 the CSF samples of BSE-infected cattle (Buschmann and Groschup, 2005). In this study,  
306 however, PrP aggregates were found in 5 out of 8 CSF samples from BSE-afflicted animals.

307 To find out whether the absence of PrP particles from CSF of BSE-infected cattle is a  
308 general phenomenon, or if the detection limit had to be improved, e.g. by concentrating the  
309 particles before application to the surface-FIDA assay, it will be necessary to optimise the  
310 sample preparation and/or the capture/probe combination. This is why we plan to perform  
311 systematic studies with CSF samples from many animals. However, at present it can be  
312 concluded that, in principle, the surface-FIDA method is capable of detecting PrP-particles in  
313 the CSF of BSE-infected cattle.

314 In the present study, we concentrated on the development of the surface-FIDA method  
315 and its basic applicability to the analysis of scrapie in hamsters and BSE in cattle. So far the  
316 number of samples has been limited and the samples were from animals in the clinical state. It  
317 is now possible to develop the surface-FIDA assay further in several directions:

- 318 (i) to further increase the sensitivity and specificity as outlined above
- 319 (ii) to use samples from animals in the preclinical stage and
- 320 (iii) samples from other organs including body fluids.

321 Surface-FIDA will possibly be useful in diagnosing other neurodegenerative diseases such  
322 as Parkinson's disease, Huntington's disease or Alzheimer's in which peptide aggregates are  
323 generated and could serve as molecular markers.

324



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331 and negative controls.

332

333

334

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410 **Figure legends**

411 **Figure 1: Model of surface-FIDA**

412

413 **Figure 2: Enrichment of bovine PrP<sup>Sc</sup> without PK.** A. purification procedure. B. Western  
414 blot analysis of the purification procedure with brain homogenate from BSE-infected cattle.  
415 C. Western blot analysis of the purification procedure with brain homogenate from non-  
416 infected cattle. gE: gram equivalents.

417

418 **Figure 3: Comparison of fluorescence bursts of one SHa PrP<sup>Sc</sup> sample and one negative**  
419 **control in different distances above the glass surface.** The fluorescence intensities are  
420 shown in the diagrams. They were detected with fluorescence labelled D13 as probe and R1  
421 as capture by surface-FIDA within 30 seconds. The measurements were carried out at heights  
422 of 0  $\mu\text{m}$  to 20  $\mu\text{m}$  above the glass surface.

423

424 **Figure 4: Surface-FIDA of purified brain homogenate of scrapie-infected hamsters and**  
425 **healthy controls.** In the histograms the mean values of burst number detected by surface-FIDA at  
426 a height of 10  $\mu\text{m}$  to 20  $\mu\text{m}$  above the glass surface are shown. PrP<sup>Sc</sup> samples from four different  
427 scrapie-infected hamsters (dark grey) and four different healthy controls (light grey) were  
428 analysed. The capture R1 and the probe combination D13 and R1 were used.

429

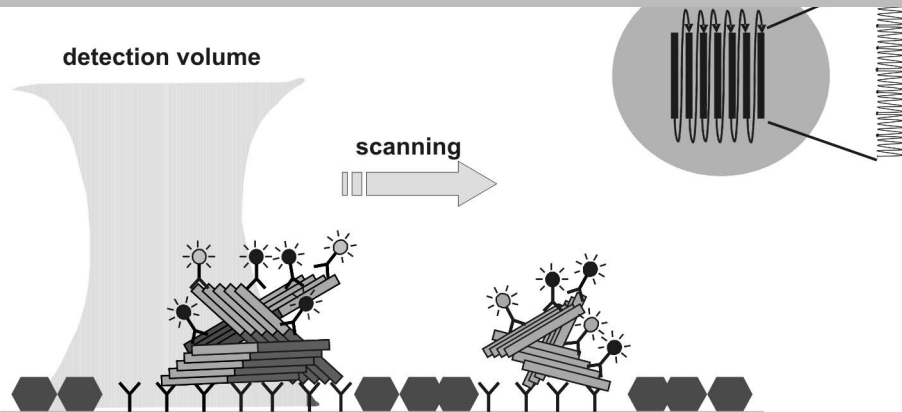
430 **Figure 5: Surface-FIDA of purified brain homogenate of BSE-infected and healthy cattle.** In  
431 the histograms the mean values of burst number detected by surface-FIDA at a height of 10  $\mu\text{m}$  to  
432 20  $\mu\text{m}$  above the glass surface are shown. BSE samples from four different BSE-infected cattle  
433 (dark grey) and four different healthy controls (light grey) were analysed. The capture Saf32 and  
434 the probe combination 12F10 and Saf32 were used.

435 **Figure 6: Surface-FIDA of BSE-infected and healthy cattle CSF with different**  
436 **capture/probe combination.** In the histograms the mean values of burst number detected within  
437 the surface-FIDA measurements at a height of 5  $\mu\text{m}$  above the glass surface are shown. CSF  
438 samples from two different BSE-infected cattle (bse1, bse2) and two different healthy controls  
439 (neg1, neg2) were analysed. As shown in the histogram, different capture/probe combinations are  
440 compared.

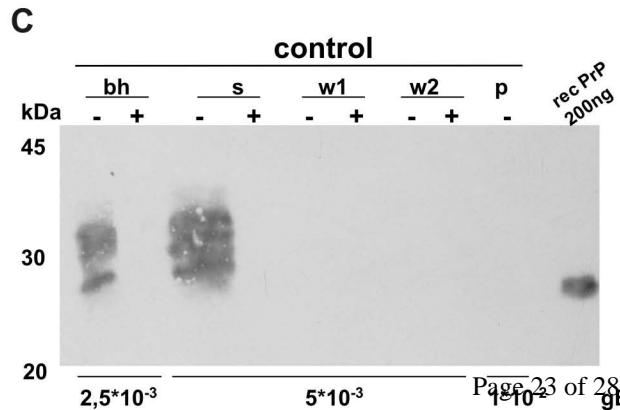
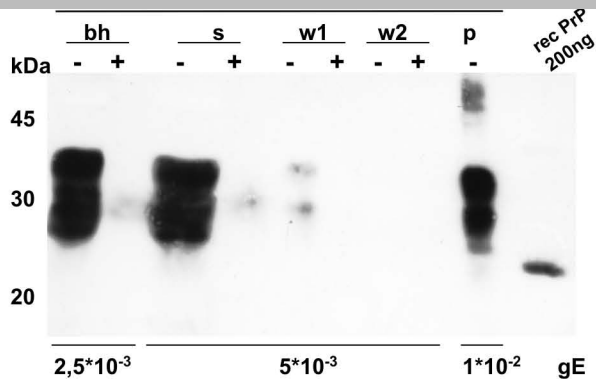
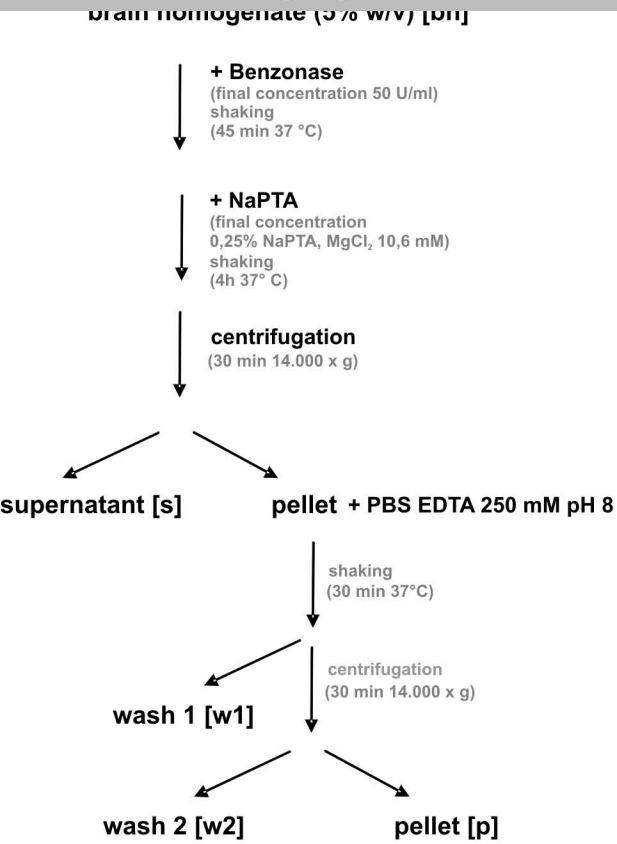
441  
442 **Figure 7: Surface-FIDA of BSE-infected and healthy cattle CSF.** In the histograms the mean  
443 values of burst number detected within the surface-FIDA measurements utilizing D18 as capture  
444 with SAF32 and 12F10 as probes are shown. CSF samples from six different BSE-infected cattle  
445 (dark grey) and six different healthy controls (light grey) were analysed. The number of peaks  
446 varied independently of the labelling grade of the probes. Here the relative differences are  
447 meaningful.

detection volume

scanning









incubation  
for 1 h at 37°C

## **capture binding**

1  $\mu$ g capture solved in PBS



incubation  
for 1 h at 37°C

## **blocking**

5% BSA solved in PBS



incubation  
for 1 h at 37°C

## **target binding**

PrP-aggregates suspended in PBS



shaking  
for 2 h at RT

## **washing steps**

3x with TBST



shaking  
for 10 min RT

## **binding of detection antibodies**

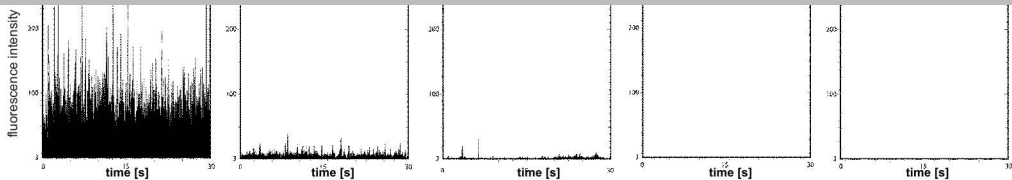
fluorescent labelled probes 0,1  $\mu$ g/ml in PBS



shaking  
for 2 h at RT

## **washing steps**

3x for 1 h with TBST

**B**