The in-situ detection of residual protein on surgical instruments: Development of the ProReveal system

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Introduction

The detection of residual proteins is considered to be a necessary step for the validation of decontamination procedures in hospital SSDs. Published national and international guidelines e.g. CFP-0101 (DH 2013) suggest that protein determinations are conducted off-instrument by either wet swabbing part of the instrument or by soaking the instrument in a detergent solution. Eluted or desorbed proteins are then determined by a variety of colorimetric chemical assays.

As previously reported (Nayuni et al 2013, Nayuni & Perrett 2013, Perrett & Nayuni 2014), problems associated with detecting residual proteins off-instrument include the inefficient desorption of proteins from the stainless steel surfaces by swabbing and the low sensitivity of the prescribed protein tests. These previous studies have demonstrated that significant amounts of adsorbed BSA or fibrinogen, (a relatively hydrophobic protein with some similar characteristics to prion proteins) can remain on the instrument after swabbing with water or detergent solutions. In addition these papers reported the poor performance of established protein tests including ninhydrin. This finding is paralleled by observations of both the poor removal of bacterial spores in bacteriological testing and the poor removal of hydrophobic drug residues in the pharmaceutical manufacture.

In situ detection

A suggested alternative approach is the in-situ detection of residual proteins on the actual instruments. Proteins adsorbed onto surfaces can be directly quantified by a number of possible techniques. Many such techniques e.g. mass spectrometry, ToC (Total organic carbon) analysis and resonance spectrometry are at present far too complex and expensive for routine use in SSDs but are used to monitor decontamination standards by the pharmaceutical industry. Another possible technique is fluorescence since it can offer considerably more sensitivity than visual or colorimetric methods as well as a much higher selectivity (specificity). Fluorescence requires intense light sources along with suitable optics to select the necessary excitation wavelength. Proteins possess native fluorescent only in the ultra-violet (UV) part of the spectrum due mainly to the presence of the amino acid tryptophan in their primary structure. The generation of intense UV-light usually requires either specialist lamps e.g. xenon or very specialist UV-lasers. These sources are expensive. Mercury (Hg) lamps, a very cheap alternative, emit intense specific lines at 254 nm and around 360 nm but unfortunately such wavelengths do not match the excitation spectra of the common blood and tissue proteins.

The use of chemical reagents that following a chemical reaction with proteins emit fluorescence was suggested in the appendix to CFP-01-01. Three Decontamination groups have been funded by the Department of Health (England) to investigate this approach for residual protein measurement both in the laboratory as well as in actual SSDs. Over the last 50 or so years, many reagents have been synthesised that can react with proteins so rendering them fluorescent with excitation spectra nearer the visible spectra. Most reagents react covalently with the amino groups in proteins, others react with thiol moieties whilst others simply bind to proteins like coloured dyes. Many were developed for the qualitative histological detection of protein classes or specific proteins in tissues. Many of these reagents are themselves fluorescent and require to be removed from the samples prior to protein measurement. In 1971, Roth observed that o-phthalaldehyde (OPA) rapidly reacts with primary amino acids in the presence of a thiol to form an isoindole with each amino acid forming a different isoindoles. The chemistry of the reaction is shown in Figure 1.
These isoindoles not only absorb in the UV but are also highly fluorescent. The most commonly employed thiol is probably mercaptoethanol (ME) but the resultant isoindoles are relatively unstable. Since proteins contain terminal amino groups OPA/ME can also react with proteins. This OPA/ME reaction is very widely used in many branches of analytical chemistry.

Instead of mercaptoethanol, Frister et al. (1994) used N,N-dimethyl-2-mercaptoethylammonium to determine the amount of protein that could be washed from surgical instruments using the detergent sodium dodecyl sulphate. The absorbance of protein isoindoles was measured at 340 nm. In 2007, McCormick and colleagues concluded that this was an effective method for validating the cleaning of medical devices. It is now included in CFPP-01-01, the British HTM’s 0101 and 2030 as well as BS EN ISO 15883:2006-Part 1.

In a number of previously published decontamination studies (e.g. Smith et al. 2005, Murdoch et al. 2006, Vassey et al 2011) Perrett developed a similar approach using an improved OPA reagent that used a significantly less noxious thiol (N-acetyl-cysteine) and fluorescent detection for much higher sensitivity towards the desorbed proteins. With aqueous samples this OPA/NAC reagent in an alkaline borate buffer reacted rapidly with protein giving μg/mL sensitivity. The isoindoles have a maximum absorbance at 335 nm but when excited at 340-380 nm they fluoresce at 440-480 nm. The fluorescent spectra of the isoindoles(s) formed with BSA are shown in Figure 2. The Stokes shift i.e. the difference between the excitation and emission wavelengths, is approx. 90 nm thereby giving low background fluorescence. In addition none of the components of this chemistry are themselves fluorescent so background fluorescence is further minimised.
Initial studies with OPA/NAC for protein detection

It therefore seemed logical to test this improved OPA/NAC reagent directly on metal surfaces although it was known that metal ions can reduce the sensitivity of the reaction. Using BSA standards spotted directly onto scalpel blades and then sprayed with OPA/NAC, fluorescence was observed using a Perkin Elmer spectrofluorimeter equipped with an X-Y scanning head attachment. This system was capable of detecting as little as 20 μg BSA and was presented by Perrett to the Department of Health decontamination working group in July 2001. However, the cuvette chamber in the spectrofluorimeter could only accommodate the smallest surgical instruments and this approach could not be progressed further at that time.

Nevertheless, the reagent had potential as a good protein derivatising agent and newer technologies suggested better approaches to detecting the emitted fluorescence. Since the required excitation wavelength for the OPA/NAC isoindoles matches with a strong line, at ca. 360 nm, found in the emission spectra of simple and cheap Hg lamps, it was considered that the fluorescence might be directly observable using a cheap hand-held Hg lamp of the type used to visualise thin layer chromatography (TLC) plates. Surgical Instruments contaminated with protein and then sprayed with OPA/NAC reagent were illuminated using such a lamp switched to emit at 360 nm and weak fluorescence was observed by naked eye under subdued normal lighting. When the instruments were illuminated in a darkroom the protein fluorophores on the instruments were more readily observed by eye. This preliminary method was shown to have potential to detect residual proteins on surgical steel in situ.

Further work was directed at improving the chemistry of the OPA/NAC reagent. NAC had been shown by a number of analytical chemistry researchers (Alvarez-Coque et al 1989, Molnar-Perl 2001) to give more stable isoindoles than ME. The reagent when formulated with ME is only stable for 2 to 2 weeks. In addition, little work had been done on its ability to derivatise proteins. The components of the reaction and the pH of the buffer have been optimised with respect to sensitivity and selectivity towards some typical proteins. The addition of a low concentration of the non-ionic detergent Brij-35 had been used to enhance the sensitivity of the basic amino acids, lysine and arginine towards OPA/ME in amino acid chromatography since ca.1980. Addition of Brij-35 to OPA/NAC was found to enhance the sensitivity towards proteins, most probably by unfolding the proteins so revealing more amino groups. However, the stability of the reagent was still only a few weeks. The addition of the dithiothreitol (a disulphide reducing agent) along with sodium EDTA to remove metal contaminants to the OPA/NAC reagent increased the stability to many months (Perrett et al. 2010a) without affecting the sensitivity. This reagent could be readily sprayed on to instruments and visualised using the TLC lamp. This formulation forms the basis of the current ProReveal spray.

Digital visualisation of fluorescent proteins

Over the last 20 years, a number of systems that could capture images of DNA and proteins separated by gel electrophoresis have been developed and sold for such biochemical and molecular biology applications. The gels are placed in a bench-top darkroom and illuminated with suitable light sources via transilluminators, simple lamps or laser beams. In the case of fluorescent derivatives, the fluorescence is captured after being filtered through appropriate optical filters. Increasingly these systems employed CCD cameras built into a darkroom enclosure to capture the emitted fluorescence. The resultant digital images can then be modified using either commercial software such as Adobe Photoshop or transformed using specialist software to outline the gel bands or spots as well as determine their optical density. Such systems can generate both qualitative and semi-quantitative data. However many such systems employed expensive lasers to excite fluorophores, such as SYPRO Ruby and fluorescein isothiocyanate (FITC) prior to visualisation.

Some systems employ Hg lamps and these appeared ideal to image the proteins post-reaction with OPA/NAC. One such gel imaging system (G:BOX) was purchased from Synoptics Health, Cambridge, U.K.. This imager was equipped with both white light and Hg lamps positioned to evenly illuminate the sample. A 450 nm interference filter was mounted in front of the CCD camera’s lens to gather the emitted fluorescence (Figure 3). The fitted trans-illuminator was replaced with a simple sample stage that could be pulled forward and onto which the instrument to be tested was placed. The test instrument was then sprayed with the OPA/NAC reagent, the door was closed and a white light image was captured. The Hg lamp was then turned on to generate a fluorescent image using a set exposure time. Figure 4 shows a superimposition of a white light image and a false coloured fluorescence image of proteins on a dental extractor. Using a
Figure 3: The original system employed a G-Box the components of the system are indicated. The current ProReveal employs many of the same system components.

convoluted combination of software in which the output file from the imaging software was imported into a low cost graphic analysis and plotting program (D-plot, HydeSoft, Vicksburg, MS, USA) 2-D and 3-D images of the fluorescence on the instruments could be created. It was also possible to calculate the area of the fluorescence contour spot as well of the volume of the fluorescent peak. By comparing the unknown peak volume to those of Bovine Serum Albumin (BSA) standards spotted onto stainless steel surfaces and then sprayed and imaged at the same time it was possible to quantify the residual protein. The overall process was time consuming but gave precise and reproducible results (Perrett et al. 2010b) that visualised the whole of the surgical instrument. It was found that to optimise sensitivity it was necessary to place the instrument on a sheet of black paper before spraying. The black paper reduced internal reflections but it was essential that it was paper made from virgin wood pulp since recycled paper now includes large amounts of fluorescent materials. Using this configuration it was possible to detect and quantify spots containing less than 1 μg of BSA protein.

Development of the ProReveal system: Instrumentation

With the clear demonstration that in situ detection of proteins on complete instruments was possible the further development of the concept was undertaken in conjunction with Synoptics Health. The ProReveal spray reagent is now prepared, tested and bottled in the authors’ laboratory for Synoptics Health, BSA is obtained from Sigma (Poole U.K). Polished stainless steel (grade 316L) tags were obtained via HealthEdge, (Bristol U.K) and used in the preparation of calibration standards.
Figure 4: The below images of a dental scaler that had been washed by hand are typical of those used in the ProReveal system. The left hand picture is the white light image, the middle image is the captured fluorescence and the right hand image shows the false colour overlay of the other two images. The residual protein is coloured green.

Figure 5: The ProReveal System showing its compact design and touch screen display.

An unplanned change resulted from an EU’s directive on mercury in the environment which is a necessary component of UV tubes, so a different lamp with lower mercury content in the tubes had to be used. This unfortunately moved the lamp’s output maximum to 368 nm, which further from the emission optimum of the OPA-protein isoindoles shown in Figure 2 and reduced the Stokes shift, leading to a slight reduction in sensitivity and higher background.

Investigation of different CCD cameras with a larger number of pixels did not improve signal-to-noise over the original camera installed. The system is now mounted in a smaller darkroom with an internal camera and a slide-out drawer where instruments to tested are placed. Closing the drawer following spraying with the ProReveal spray reagent automatically starts the control and analysis software. The present form of the ProReveal is shown in Figure 5 and its specification is in the table.
Development of the ProReveal system: Software

The previous method of generating images and the method of protein calculation was convoluted and unsuitable for use as an instrument planned to run in routine environments. New software was therefore developed that combined all aspects of the system into one screen. This software controls the lighting and the camera, to capture the fluorescent images, and perform image analysis. The process is started by closing the drawer, which is locked electronically for the duration of the sequence. A white light image is captured, which is then analysed to identify the outline of the surgical instrument. The fluorescent lamp is triggered and is allowed to stabilise for a few seconds before capturing a long exposure of the emission from the derivatised protein that is analysed to measure the quantity of protein on the surgical instrument. This entire imaging process takes 2 to 4 minutes depending on the software settings. A false coloured image showing protein residues is then displayed along with the quantity of protein. A 3-D representation of emission density across the surface of the surgical instrument may be rotated on screen in order to inspect and locate protein residues more clearly. The SSD manager can select from a range of measurement options e.g. μg total protein per instrument, μg/cm² of the instrument. The software is presented at two levels, a simple screen presenting the basic numerical outputs (Figure 6) and a more complex control software that is only available to an administrator. It is also possible to trigger a warning if the amount of protein residues exceeds a pre-set threshold. In order to generate quantitative data, the instrument needs to be calibrated against known amounts of a standard protein (BSA) dried on to stainless tokens. The response of the present system to BSA standards is linear from 0.1 μg to 50 μg BSA (Figure 7) per spot and the limit of detection is 50 ng per spot. A typical image from a washed instrument is shown in Figure 8. On completion all data is permanently stored either on the PC or externally within the organisation.

Figure 6: The ProReveal screen output in inspection mode showing a 2-D image with the residual protein shown in yellow and a numerical value for the total protein shown.
Questions have rightly been raised about returning instruments following in situ testing to clinical use after they have been re-washed. This matter has been discussed at the Department of Health (England) on a number of occasions and a number of points are relevant here. Only very low concentrations of the fluorescent reagents e.g. OPA/NAC, FITC and SYPRO Ruby used by the three Department of Health sponsored research groups are placed on instruments. All state that at this time a re-wash of the instrument using standard processes is required before further use. In the case of the ProReveal spray reagent all fluorescent proteins were removed to our limit of detection by a routine re-wash in an alkaline detergent. We advise that all instruments sprayed with the OPA/NAC reagent must be re-washed to remove any reagent residues.

**Figure 7:** A 3-D fluorescence image of BSA standard peaks adsorbed onto a stainless steel tag post reaction with OPA/NAC. The amounts of BSA ranges (left to right) from 125 ng to 1 µg per spot. The lower figures show the linearity of the peak volumes.
The chemical components in the ProReveal reagent are non-fluorescent and when instruments are re-sprayed with an amino acid solution (a test for the OPA/NAC reagent) no fluorescence is observed. There is very little in the way of toxicity data in the published literature at the concentrations employed. The actual amounts of chemicals in 1 mL of the reagent, which is more than the typical volume sprayed on to most instruments, is negligible and is easily washed off with water. NAC is used clinically to treat both mucolytic disorders and paracetamol poisoning. In mice NAC toxicity is only found at doses above 20g per day, which is equivalent to the mouse drinking 13 litres of reagent daily. Formulations of OPA/ME for amino acid analysis and protein determination have been sold for many years by a number of companies yet the supplied data sheets state only routine precautions. There are few studies on OPA itself in animal models but again toxicity was only at doses far in excess of the concentration (0.01 % w/v) in the reagent. The HSE reported a LD$_{50}$ for a single oral dose of technical grade OPA in rats to be 121 mg/Kg and when dermally applied to rabbits the LD$_{50}$ was > 2g/kg (HSE 2003). This report on OPA's low toxicity confirms a much earlier report in mice (LD$_{50}$ = 27 mg/kg) by Caujolle et al. (1956).

All such studies should be seen against the current levels of non-self proteins that can be introduced into patients from poorly cleaned instruments and the residuals of detergents etc. on them for which there appears to be very little published data. The Department of Health working group were informed that approval of a clinical scientist is sufficient to permit the use of all such reagents in the SSDs.

**Advantages of in situ detection using ProReveal**

The system offers a number of immediate advantages over the swabbing techniques usually employed in SSDs. Firstly, for the majority of instruments the whole of the instruments can be inspected for residual protein using a simple and partially automated process. Other than reflection off some surfaces, which is readily distinguished from protein residues, there is no fluorescent background seen on the images. The reagent not only works on flat surfaces but also on difficult to swab areas such as serrated edges and teeth, curled screws and nuts and to some degree box joints, The visual information in the 3-D images can be very informative about residues trapped on instrument components. Secondly the data generated is quantitative since the system is calibrated against BSA. The older methods usually give only qualitative information, such as the visual appearance of a swab. Permanently stored quantitation aids good practice. Finally the sensitivity of the system is 2 orders of magnitude better than the current approaches named in the CFPP 0101 and HTM 0101 documents. The supplied standard

![Figure 8](image-url): A typical study using ProReveal of two eye instruments washed using two different detergents. The right hand image shows the washing achieved using an enzymatic detergent and the left hand follows washing with an alkaline detergent.
range is from a 0.125 μg spot to a 8 μg spot giving a spot density of approximately 1 ng/mm² to 80 ng/mm² values that equate to protein coverage found on many instruments. The limit of detection for a single spot of BSA placed onto a stainless steel token is 50 ng. A set of standards peaks used for a research application along with the plotted standard curve of the fluorescent peak areas is shown in Figure 7.

**Actual and envisaged uses of ProReveal**

To date we have mainly employed the ProReveal system to gather data for the validation of AWDs in routine operation in various hospitals in Britain as part of a major Department of Health funded study. The ability to generate robust quantitative results on topics such as moist versus dry and alkaline detergent versus enzymatic detergents has proved valuable in understanding the processes involved in actual SSDs. These results will be published in due course. We have also been able to help SSDs in their choice of detergents and/or AWDs by investigating the performance of possible combinations against standard loads. For example, we have shown that not all shelf positions in AWDs wash to the same efficiency (Figure 9). Therefore, we envisage that the system could be used by manufacturers of AWDs of detergents for developing improved systems and chemistries. The same applies to surgical instrument manufacturers to develop improved designs with fewer details that could trap proteins. ProReveal was originally envisaged for routine QC and QA in SSDs but uptake in this area awaits more definitive guidance from national and international bodies on the levels of proteins permissible on re-usable surgical

**Figure 9:** A ProReveal study showing the effect of shelf position in the same AWD on the removal of brain homogenate dried onto stainless steel tags. An alkaline detergent was in use. Top image show a test tag from the top shelf with 43 ± 5 % residual protein, the middle image (middle shelf) shows 93 ± 14 % residual protein and the lower image (bottom shelf) shows 39 ± 13 % residual protein.
instruments to reduce risk and the units by which this can be expressed. Dr Nigel Tomlinson suggested, in his recent lecture to the IDSc conference in Blackpool that to minimise risk, especially that from vCJD where we are now aware that some 1 in 2000 of the UK population are carriers (Gill et al. 2013). 5 μg of residual proteins per entire instrument should be our goal although even lower levels of residual might be needed for high risk instruments such as those used in neurosurgery. Our studies have shown that these levels of residual protein can be routinely achieved even with the current sensitivity of the ProReveal system in current SSDs.

Conclusions

A simple, high sensitivity in situ system useable in SSDs has been developed and is now available. Although we still await definitive guidance from DH (England) on the levels of proteins permissible on re-usable surgical instruments the present ProReveal system is at least 100x more sensitive than present tests currently available and this should more than meet any new requirements set by the national bodies.

Table Specification of ProReveal imaging system

<table>
<thead>
<tr>
<th>Camera</th>
<th>Cooled CCD</th>
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<tbody>
<tr>
<td>Sensor</td>
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<tr>
<td>Lens</td>
<td>Max f/1.2 but set at f/2.8</td>
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<tr>
<td>Maximum Specimen size</td>
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<tr>
<td>Lamp</td>
<td>2 banks of white light diodes</td>
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<td>Emission Filter</td>
<td>interference type passing 450 nm</td>
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<td>Dimensions (W x H x D) cm</td>
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References


