Highly sensitive rapid fluorescence detection of protein residues on surgical instruments

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Abstract. There is a risk of contamination of surgical instruments by infectious protein residues, in particular, prions which are the agents for Creutzfeldt-Jakob Disease in humans. They are exceptionally resistant to conventional sterilization, therefore it is important to detect their presence as contaminants so that alternative cleaning procedures can be applied. We describe the development of an optimized detection system for fluorescently labelled protein, suitable for in-hospital use. We show that under optimum conditions the technique can detect ~10 attomole/cm² with a scan speed of ~3-10 cm²/s of the test instrument’s surface. A theoretical analysis and experimental measurements will be discussed.

1. Introduction
The risk of cross-contamination of surgical instruments by infectious protein residues is of current public and professional concern. Prions are misshapen or abnormally folded forms of the natural PrP protein, and are of special concern because they are exceptionally resistant to inactivation by conventional chemical and physical decontamination procedures [1]. This makes it extremely important to develop routine techniques for the detection of such contaminants in hospital sterilization departments. Detection of fluorescence from fluorescent labels attached to proteinaceous material was suggested for this purpose [2]. It is known that under optimum conditions, in the research laboratory, the detection of single molecules is possible through this technique [3], but this experience is not applicable to the mass screening of surgical instruments because the procedure is extremely time-consuming. In this work we report the development of an optimized detection system for fluorescently labelled protein, suitable for in-hospital use.

2. Theoretical background
Prior to the optimization of the fluorescence detection system it would be useful to understand and to estimate the fundamental detection limit for such system. The treatment of this problem must include the analysis of fluorescence excitation, collection and detection stages.

By its nature fluorescence is the emission from the same electronic state of a molecule as initially reached by the excitation process. The difference between energies of the absorbed and emitted photons is due to vibrational deactivation of the excited energy level within the potential curve of the upper electronic state. The shift between absorption and emission spectra usually is rather small. An example of such spectra of the fluorophore we use for protein labeling is shown in figure1.

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The fluorescence signal power, \( P_F \), is usually estimated as [4]

\[
P_F \equiv \frac{I_0 S}{\eta \sigma D}
\]

where \( I_0 \) is the excitation radiation power density, \( S \) is the excitation spot area, \( l \) is the thickness of the fluorescently labelled protein layer, \( \eta \) is the quantum efficiency, \( \sigma \) is the absorption cross section, and \( D \) is uniform (bulk) concentration of the fluorophore. In our case equation (1) is valid for considerable surface concentrations of labelled protein, \( N \), that is of order or above \(~10^{15} \text{ cm}^{-2} \equiv \sim 10^{-9} \text{ mole/cm}^2 \). This is because of huge difference between the area size of a typical protein molecule (\( \geq 10^{-11} \text{ cm}^2 \)) and the absorption cross section of the fluorophore (\( \leq 10^{-15} \text{ cm}^2 \)). For smaller concentrations of protein molecules in a layer \( P_F \) becomes to be independent of \( l \), and the proper equation reads

\[
P_F \equiv \frac{I_0 \eta \sigma N S}{2}
\]

Here we supposed that only one molecule of fluorophore is attached to label a protein molecule, otherwise \( P_F \) must be increased accordingly.

Because fluorescence emission is a spontaneous process, the radiation emitted by an individual molecule is distributed uniformly in space (i.e. in a solid angle of \( 4\pi \)). Nonuniformity of this distribution is associated usually with existence of neighbors: other molecules, crystal structures, substrate, etc. In our case, where small amounts of fluorophore are deposited on a metal substrate, the radiation emitted towards the substrate will be reflected back. Because the reflectivity of metals in the visible wavelength region is usually not less than \(~80\% \) and we can ignore absorption losses in the thin protein layer, the full power of fluorescent radiation, \( P_F \), is uniformly distributed in the half-space above the metal substrate. If all this power were to be collected and directed to a detector by an optical system, it would represent the highest possible efficiency of the detection system. In practice a real optical system may collect only a portion of this power. The amount of fluorescent radiation power, which may be delivered to a detector with the receiving aperture bigger than the spot size of an arriving beam is then defined by the equation

\[
P_1 = P_F \Psi_{\text{eff}}/2\pi = P_F \left( \frac{1}{F^2} \right)^2 = \alpha P_F
\]

where \( \Psi_{\text{eff}} = \Psi_0 \left( 1 - \frac{r}{R} \right)^2 \) is the effective solid angle of the lens, which collects a radiation emitted from a spot of radius \( r \) at a surface placed at focal plane of the lens, \( \Psi_0 = \pi R^2/F^2 = 2\pi(NA)^2 \), \( F \), \( R \) and
NA are the focal length, radius of aperture and numerical aperture of the lens respectively. For reasonable values of the ratio $r/R$ of $\sim 0.1-0.05$ and $NA = 0.5$ equation (3) gives $P_1 \approx (0.1 - 0.2) P_F$.

3. Experimental set up

The arrangement for fluorescence detection of the protein residues is shown in Figure 2. We use for excitation of the fluorescence a LS-475 blue LED (Ocean Optics BV) (1), which provides CW radiation in the wavelength region around 475 nm of nominally $\sim 50 \mu W$ power with a 600 $\mu m$ core diameter optical fibre. The radiation at the fibre output is collimated by a lens (2) and directed to the sample (3) surface by a dichroic mirror (4) through a lens (5). This optical system allows variation of the irradiation spot diameter in the range of 1-5 mm. In the majority of our experiments the spot size was $\sim 2 \text{ mm}$ and the radiation power at the sample surface, $P_0$, of $\sim 25 \mu W$. A combination of two identical high NA ($\sim 0.5$) lenses ((5) and (6)) with the aperture diameter 25 mm is used for collecting the fluorescence signal and coupling it to a fibre bundle (7) with radiation transmittance $\sim 60\%$. The bundle delivers the fluorescence radiation to the cathode of the PMT, (8). The diameter of the bundle ends is $\sim 3 \text{ mm}$ ensuring that maximum emitted fluorescence is delivered to the PMT. Band-pass filters (9) and (10) are used to reduce the amount of the Raman scattered light generated in the 600 $\mu m$ fibre [2] and to separate the fluorescence signal from the excitation radiation respectively. The PMT based photosensor module H5784-01 (Hamamatsu) has an effective diameter of the cathode 8 mm and a radiant sensitivity of $\sim 20 \text{ V/nW} (\lambda \cong 530 \text{ nm})$. The signal from photosensor is registered by a digital oscilloscope.

![Figure 2](image_url)

Figure 2. Experimental set up. (1) pigtailed LED, (2), (5) and (6) lenses, (3) sample, (4) dichroic mirror, (7) fibre bundle, (8) photosensor, (9) and (10) band-pass filters.

A typical fluorescence emission signal at the oscilloscope screen is shown in Figure 3. For the demonstration goals the excitation radiation beam was mechanically chopped with modulation frequency of $\sim 200 \text{ Hz}$. The signal comprises a pronounced DC component with a considerable irregularly spiked component superimposed (Figure 3a). To reduce the high frequency spiked component and increase the accuracy of signal amplitude reading a smoothing capacitor of $\sim 45 \mu F$ was connected parallel to the oscilloscope input. A typical smoothed signal is presented in Figure 3b. The estimated accuracy of signal amplitude measurement in this case is $\sim 3-5\%$. 

4. Measurement results

4.1. Angular distribution of the fluorescence emission
The feature of principal importance for the effective collection of the fluorescence radiation and for the practical use of the scheme presented in Figure 2, is uniform angular distribution of the emitted radiation. To verify this we investigated experimentally the dependence of the fluorescence signal on the angle between the normal to the sample surface and the optical axis of our radiation collection scheme. For this purpose a sample was placed on a rotation stage with angle measurable to ±0.5°. The results of measurements, presented in Figure 4, explicitly demonstrate the absence of essential angular dependence for the angles of the sample surface tilts from 0 to ~24°.

4.2. Detection of labelled protein
To characterize the sensitivity of the experimental scheme presented above, the dependence of the fluorescent signal from fluorescently labelled bovine serum albumin (BSA) was investigated as a function of the concentration of BSA. To obtain quantitative estimates of the sensitivity 5 μl volumes of various concentrations of FITC-labelled BSA, in water, were deposited on surgical steel substrates. The water was allowed to evaporate producing a thin layer of fluorescently labelled protein on the substrate surface. Using this method we were able to prepare the samples with concentration of fluorophore from ≥ 10 pmole to ~3 fmole per a spot of ~3-4 mm diameter on the substrate.
The results of measurements, which are presented in Figure 5 by dots, demonstrate a linear (in log-log scale) dependence of the fluorescence signal amplitude on the labelled BSA amount through the whole range of BSA amount investigated.

![Figure 5](image.png)

**Figure 5.** Fluorescence signal versus amount of FITC-labelled BSA on surgical steel discs (dots), linear extrapolation of the experimental data (solid line) and the maximal amplitude of the scattered excitation radiation (dashed line).

4.3. Scattered signal
Among the factors which define the minimum detectable fluorescence signal in our system is the power of the reflected and scattered excitation light. The level of this signal at the output of our PMT was measured for several blank samples of surgical steel discs. The amplitude of the experimentally measured signal varied from sample to sample between ~0.5 and ~2 mV and for none of these did this signal exceed 2 mV.

5. Discussion
The results of measurements presented in Figure 5 and in section 4.3 allow us to conclude that our system is capable of detecting ~0.15 fmole per illumination spot of ~2 mm diameter that is the detectable surface concentration is ~5 fmole/cm².

Let us compare this value with that following from Equations (2) and (3). Substituting \( I_0 = \frac{P_0}{S} = 10^{-3} \text{ W/cm}^2 \), \( \sigma = 10^{-15} \text{ cm}^2 \), \( \eta \equiv 0.7 \) [2], \( \text{NA} = 0.5 \), \( r = 1 \text{ mm} \), and \( R = 12.5 \text{ mm} \) one obtains a detected fluorescent power of \( P_1 = 1.7 \times 10^{-21} \text{ W} \). If we suppose the minimum measurable signal voltage to be of ~2 mV (signal-to-noise ratio of ~1, see Figure 5) the minimum detectable radiation power with our photosensor, \( P_{\text{min}} \), would be \( 10^{-13} \text{ W} \). Comparing this value with the estimated value of \( P_1 \) one can obtain the minimum detectable \( N_{\text{min}} \) to be ~0.1 fmole/cm², which is ~50 times smaller than we realized experimentally.

This difference may be attributed to several reasons. Actually, there might be some radiation losses in our system, which are unaccounted in our estimates. We suppose that this may give us a factor of ~2, but not more. The most considerable contribution to the observed reduction of real sensitivity compared to that which follows from the theory presented above may be associated with the statistics of excitation of small amount of randomly distributed fluorophore molecules by the flow of excitation photons, the density of which is less than one per molecule.

The only way to reduce this latter effect is to increase the illumination intensity. The highest possible illumination intensity may be estimated as the saturation intensity for the absorption transition in a molecule, \( I_0 \).
IS = \frac{h\nu}{\sigma \tau} \quad (4)

where \( h\nu \) is the photon energy of the absorption transition, \( \sigma \) is the cross section of the transition and \( \tau \) is its decay time. Substituting \( h\nu \approx 4.10^{-19} \text{ J} \), \( \tau \approx 4 \text{ ns} \) [2], and \( \sigma \approx 10^{-15} \text{ cm}^2 \) one obtains \( IS \approx \frac{h\nu}{\sigma \tau} \approx 10^{5} \text{ W/cm}^2 \). The illumination with such intensity means that at least one photon will be delivered to each individual atomic transition during the excitation pulse. In this case the probability of excitation of all fluorophore molecules in the spot will be the highest possible, that is of order of 1.

With such excitation intensity the pulse duration must be no longer than \( \tau \) to prevent photobleaching and overheating of the fluorophore. The only light source able to deliver such characteristics is a laser. Our survey of laser sources emitting radiation wavelength \( \sim 0.49\pm 0.05 \mu \text{m} \) with pulse duration \( \sim 4 \text{ ns} \) and pulse energy of \( \sim 50-100 \mu \text{J} \) has revealed that only two systems are capable to deliver required parameters of emission: dye and OPO lasers pumped by third harmonic of Nd:YAG laser emission.

The number of photons to be registered by a modern PMT in the analogue mode is of \( \sim 10^6 \) during a of \( \sim 4 \text{ ns} \) pulse. Then, if we suppose that every molecule of fluorophore will emit only one photon during an excitation-emission event, the number of molecules, \( N \), detectable by the proposed system from a spot size of \( \sim 1 \text{ cm}^2 \) must be \( \sim \left( \frac{10^6}{\alpha \eta} \right) \approx 10^7 \). So, we expect the sensitivity of the system \( \sim 10 \text{ attomole/cm}^2 \) per one laser pulse of \( \sim 4 \text{ ns} \) duration.

Such characteristics of the detection system allow us to estimate the achievable level of the surface inspection speed for the future scanning instrument. For an illumination area of \( \sim 0.3 \text{ cm}^2 \), which corresponds to the area of \( 6 \text{ mm} \) diameter at the cathode of the PMT, using a laser providing \( \sim 100 \mu \text{J} \) in \( 4 \text{ ns} \) pulses with a repetition rate of \( \sim 30 \text{ Hz} \) the estimated scan speed is expected to be \( \sim 10 \text{ cm}^2/\text{s} \).

6. Conclusions
Detection of fluorescence emission from a low surface concentration of FITC-labelled BSA is a highly sensitive and efficient method for detecting residues of infectious labelled protein on surgical instruments. Experimental studies have verified the linearity of the emission power as a function of the amount of BSA in the range of its concentration from \( \sim 30 \) to \( \sim 0.1 \text{ pmole/cm}^2 \). The linear extrapolation of the obtained dependence to the minimum detectable signal, defined by the noise, has shown that minimum detectable surface concentration of BSA would be \( \sim 5 \text{ fmole/cm}^2 \) with a low power CW LED as the source of the excitation radiation. The use of a higher power pulsed laser for these purposes would increase the detection sensitivity limit to \( \sim 10 \text{ attomole/cm}^2 \) and achieve an instrument scanning speed up to \( \sim 10 \text{ cm}^2/\text{s} \).

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