Optical design and development of a cost-efficient, compact, super-sensitive scanning confocal fluorescence microscope

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ABSTRACT

The effective detection of very low abundant biomarkers can enable fast diagnosis of many severe and disabling diseases (e.g. Alzheimer’s Disease) at an early stage. To develop a cost-efficient, super-sensitive optical fluorescence detection microscope, we have proposed an optical modelling approach to predict the signal-noise-ratio that considers various noise sources introduced by the components of the detection system. After the optimal design is identified, a tolerance analysis regarding typical perturbations is performed for further mechanical design and assembly. Finally, experiments have demonstrated a limit of detection at a low abundant concentration reaching 0.05 pmol/ml.

Keywords: fluorescence microscopy, laser induced fluorescence, optical scatter modelling, Alzheimer’s Disease, confocal scanning, tolerance analysis, optical design

1. INTRODUCTION

The early diagnosis of fatal diseases (Alzheimer Diseases, cancers, etc.) relies on the detection of biomarkers at very low abundance (<1 pM)1. In clinical practice, the mainstream methods such as standard Enzyme-Linked Immunosorbent Assay (ELISA) and chemiluminescence (CL), require around 100μl sample volumes and 0.1-1 picomole protein biomarkers, thus can fail when detecting low-abundant biomarker for early disease diagnosis2-4. Various approaches have been reported to improve the limit of detection, such as photonic crystal surface5,6 and nano-structured surface enhancement7; however, they typically rely on high-specialized or time-consuming sample preparation. Our previous work has led to an innovative technique called “droplet split-and-stack” (DSS)8-9, which significantly reduces the droplet diameter down to a few microns and improve the sample concentration by dozens of times. Based on this technique, we aim to develop a simplified fluorescence device to substitute the sophisticated, bulky commercial confocal fluorescence detection instruments. When designing such a portable fluorescence detection system using mass-produced optical components, the optical characteristics should be carefully examined to suit the core requirements of high diagnostic accuracy and sensitivity. This study developed a simplified confocal fluorescence detection system using off-the-shelf components that can measure the fluorescent molecules below 0.1 femtomole.

As we know signal-noise-ratio (SNR) is the most important criterion for supersensitive detection, which is the ratio of unbiased signal amplitude to the standard deviation of time-domain noise. An SNR of at least 3.3 is required for a reliable measurement10. Although many researchers have taken efforts to improve the signal collection efficiency with high numeric (NA) apertures11 or supercritical angle fluorescence12, fewer attentions have been paid to noise reduction13. Except for the detector readout noise and photon noise, the noise from scattered stray laser light and autofluorescence of the sample slide or cuvette is usually ignored in most fluorescence optical system designs14. The laser noise in this work is mainly from laser scattering within the light path, which causes stray light of large incident angles that passes through the filters and reaches the detector. Due to absence of proper modeling, this issue has either been overlooked or too complicated to give a rapid yet effective results.

In this work, we carefully revisited the ABg scattering model regarding different optical surfaces, and then measured the scattering of two optical surfaces and demonstrated the presented theoretical ABg model by well-matched data
fitting. A full SNR calculation over various optical fluorescence detection configurations is proposed to further quantify the system performance. Then, we have proposed both slanted- and perpendicular-illumination for an epi-illumination confocal fluorescence design. The proposed scattering modeling and SNR calculation program are applied to predict the performance of various illumination configurations and filter combinations in confocal fluorescence detection, providing a rapid way to evaluate the system SNR without time-consuming fabrication and costly prototyping. Based on the SNR simulation results, one of the simulated systems was built and experiments were made to validate the effectiveness of the proposed method. Experimental results have demonstrated that the as-built system can reach a limit of detection at a low abundant concentration.

2. OPTICAL CONSIDERATIONS

2.1 Optical design of confocal fluorescence detection

Confocal microscopy uses a spatial pinhole to block out the signal that is out of focus, ensuring a high SNR. A conventional confocal microscope comprises of a laser beam, filters, a sample slide, collection optics, a pinhole, a tube lens and a detector using a Photomultiplier Tube (PMT). It is very typical to use perpendicular illumination in most fluorescence microscopes, as illustrated in Fig. 1(a). An alternative approach with slanted-illumination sketch can be seen in Fig. 1(b) using a freeform beam shaper to generate a top-hat beam.

![Image](image.png)

From the perspective of improving collection efficiency, different design strategies of collection optics have been investigated, by using a compound parabolic concentrator (CPC), a gradient-index (GRIN) lens and/or a microscopic objective, as seen in Fig. 1(c)-(e). A CPC usually has the highest collection efficiency with a very limited working distance and field of view. A GRIN lens is super compact, but it has a relatively low collection efficiency and quite short working distance. A microscope objective is certainly good for extended targets, but the high NA requirement also implies a very short working distance, and it is usually quite expensive. Increasing the number of lenses can improve the imaging quality, but sometimes a single aspheric lens can also achieve a relatively large numerical aperture (NA) while keeping a quite practical working distance for tiny targets, shown in Fig. 1(f).

In terms of noise reduction, the laser light reflected and/or scattered back to the emission path can be a source of considerable amount of noise when the fluorescence signal is extremely weak, e.g. about 10^{-10} of laser source intensity. Most fluorescence microscope designs have ignored this issue as either the sample volume is sufficient or the sample concentration is high; however, in a supersensitive detection (e.g. 1pg/mL for 1μL sample solution), the laser scattered noise presents a bottleneck. This is also a case with the confocal microscopy designs shown in Fig 1(a) and (b). To improve the collected fluorescence signal quality, the scattered laser noise of these two illumination configurations were evaluated and minimized as discussed in the following sections.
2.2 Optical scatter modelling

Light scattering is ubiquitous, especially on rough surfaces, which are frequently used in point-of-care assays. Here, we introduced a generalized scattering model for fluorescence detection with laser illumination on a sample slide as shown in Fig.2.

Figure 2. Scattering model on a thin sample slide where the incident beam propagates as transmitted, specular reflected and scattered light beams

The scattered beam out of the specular reflection can be viewed as the result of fluctuations over the sample surface depending on the surface waviness. The root mean square (RMS) roughness over rectangular region $L_x \times L_y$ is defined as

$$\sigma_s = \left( \lim_{L_x \to \infty} \frac{1}{L_x L_y} \int_0^{L_x} \int_0^{L_y} \left[ z(x,y) - \bar{z} \right] dxdy \right)^{1/2}$$

(1)

The total integrated scatter (TIS) is the ratio of scattered power $P_s$ over the total reflected power $P_r$, which is directly related to surface roughness by

$$\text{TIS} = \frac{P_s}{P_r} = \frac{P_s}{R.P} = \left( \frac{2 \pi \Delta n \sigma_s \cos \theta_s}{\lambda} \right)^2$$

(2)

where, $R$ is the reflection rate, $\Delta n$ is the refractive index difference, and $\lambda$ is the incident wavelength. More details regarding the correlation over incident angles and surface roughness have been investigated by J. Harvey et al. 21.

A universal metric to quantify scattering features is the Bilateral Scatter Distribution Function (BSDF) that accounts for the specified surface radiance per unit solid angle $\Omega_s$

$$\text{BSDF} = \frac{P_s / \Omega_s}{P_r \cos \theta_s}, \text{sr}^{-1}$$

(3)

BSDF is generally applicable to both bulk scatter and surface scatter. It is also often referred as another similar term angle-resolved scatter (ARS), where the cosine term is dropped. The Rayleigh-Rice theory further relates ARS to surface power spectral density function (PSD) by

$$\text{ARS} = \frac{16 \pi^2}{\lambda^4} \cos \theta_s \cos^2 \theta_s Q.PSD(f)$$

(4)

where, $Q$ is the angle dependent polarization reflectance, determined by the interface dielectric function as well as the illumination and detection conditions. This theory allows a non-contact metrology using light scattering for characterizing high-precision surface roughness, especially for anisotropic optical surfaces, such as gratings and diamond-turned surfaces 23-24.

Most optical design software, e.g. Zemax, provides several options to model surface scatter by using different BSDF models, such as Harvey 25, Lambertian, Gaussian, tabulated BSDF data and ABg model 26. ABg model is suitable for
various isotropic optical surfaces which needs only three parameters, thus very efficient regarding the considerable ray tracing in non-imaging optics, such as stray light analysis and low-abundance signal detection. It is defined as

\[
BSDF(\beta - \beta_0) = \frac{A}{B + (\beta - \beta_0)^g}, \quad sr^{-1}
\]

where \( \beta = \sin(\theta_i) \), \( \beta_0 = \sin(\theta_{\text{specular}}) \), \( \theta_{\text{specular}} = \theta_i \) and \( A, B, g \) are the modeling parameters. Some suitable parameters for polished mirrors and lens surfaces can be found in Ref. \(^{27}\).

In order to quantify the scattering induced laser noise, we need to integrate the intensity over the acceptance angle \( \theta \) of the collection optics

\[
\text{TIS}(\theta) = \int_0^{2\pi} \int_0^\theta BSDF(\beta - \beta_0) \cos \theta \, d\Omega,
\]

\[
= 2\pi \int_0^\theta \frac{A}{B + (\sin \theta_i - \sin \theta)^g} \cos \theta_i \sin \theta \, d\theta
\]

(6)

Compared to Eq. (2) for perpendicular illumination, this equation gives a more generalized way to calculate the TIS with different incident angles. The scatter yield is further defined as the ratio of the scattered laser light on the detector to the input power

\[
\eta_{\text{sc}} = \frac{P_{\text{sc}}}{P_i} = \text{TIS}(\theta).R
\]

(7)

3. SIMULATIONS AND TOLERANCE ANALYSIS

3.1 SNR calculation procedures

To evaluate the SNR performance of various confocal fluorescence detection configurations, we need to quantify both the generated fluorescence signal and all types of noise on the photodetector. In terms of laser illumination, the laser power \( P_0 \) reaching the sample is calculated by

\[
P_0(\lambda) = \eta_L P_L \Phi_L(\lambda) T_{\text{ex}}(\lambda) T_{\text{op}}(\lambda) \rho_{\text{th}}(\lambda)
\]

(8)

where, \( P_L \) is the total laser power (W), \( \eta_L \) is the percentage of laser shining on the desired sample spot, \( \Phi_L \) is normalized laser spectrum, \( T_{\text{ex}}, T_{\text{op}} \) are transmission spectra of the excitation filter and other optics in the excitation light path. \( \rho_{\text{th}} \) is the reflection spectrum of the dichroic filter; if no dichroic filter, \( \rho_{\text{th}} = 1 \).

The laser induced fluorescence signal is proportional to the total absorbed laser power. Based on Beer-Lambert law \(^{28}\), the absorbed power by the sample is

\[
P_{\text{abs}}(\lambda) = P_0(\lambda) - P_a(\lambda) = P_0(\lambda) \left(1 - e^{-\varepsilon(\lambda) \cdot c \cdot d}\right)
\]

(9)

where, \( P_0(\lambda) \) and \( P_a(\lambda) \) are the incident and outgoing laser radiant flux (Watt), \( c \) is molar concentration (M) and \( \varepsilon(\lambda) \) is extinction coefficient (M\(^{-1}\)cm\(^{-1}\)), \( d \) is the sample thickness.

Provided a low concentration, the absorbed light power is approximately obtained by

\[
P_{\text{abs}}(\lambda) \approx P_0(\lambda) \varepsilon(\lambda) \cdot c \cdot d
\]

(10)

Note that, the linear approximation is not valid at high dye concentration due to quenching. The extinction coefficient can be also denoted by \(^{29}\)

\[
\varepsilon(\lambda) = \log(10) \cdot \text{DMAC} \cdot \Phi_A(\lambda)
\]

(11)
where, DMAC stands for the maximum decadic molar absorption coefficient and is a standard parameter for fluorophore dyes. $\Phi_\lambda$ is the normalized fluorophore absorption spectrum. The total absorbed power is

$$\hat{P}_{\text{abs}} = \int P_{\text{abs}}(\lambda) d\lambda = \log(10) \cdot \text{DMAC} \cdot c \cdot d \int P_e(\lambda) \Phi_\lambda(\lambda) d\lambda$$

Assume the fluorescence light is emitted like Lambertian in all direction, the effective signal that is collected by the emission optics and then reaches the detector is

$$S = \int P_{\text{em}}(\lambda) d\lambda = \frac{\Omega}{4\pi} \cdot \eta_p \cdot \hat{P}_{\text{abs}} \int \Phi_e(\lambda) T_{\text{em}}(\lambda) T_{\text{opt1}}(\lambda) T_{\text{opt2}}(\lambda) T_{\text{det}}(\lambda) \cdot \eta_D(\lambda) d\lambda$$

$$\text{SNR} = \frac{S}{N_L + N_{\text{AF}} + N_{\text{rd}}}$$

### 3.2 Quantify laser scatter noise on two typical slides

To quantify the scatter noise yield, we have built a simplified system in Zemax using two aspheric lenses (Thorlabs ACL25416U, AL2550) for the collection optics and the tube lens respectively, together with a set of excitation, emission and dichroic filters (Semrock LED-Cy5-A-000) and a pinhole (Thorlabs P200D, different sizes available). This design possesses a high-efficiency NA of 0.79 while keeping a reasonable working distance of about 7.3mm. Two typical sample slides are selected, ONCYTE (Grace bio-labs, 705278) and Super Amine (Arrayit, SMM2), representing a rough surface typically used in PoC assays and a highly polished smooth slide respectively.

The scattering properties of the two slides were characterized by scatterometer (REFLET Bench, Light Tec), and the results are plotted in Fig. 3. In general, the ONCYTE slide scatters like a Lambertian with slightly higher reflection over the specular angle, while the SuperAmine performs like a smooth glass plate, which has a specular reflection direction with the majority of the light energy.
Figure 3. Measured BRDF characteristics of (a) ONCYTE slide and (b) SuperAmine slide from different incident angles 0, 30 and 60 respectively. Note: the small bumps are due to the detector blocking the light source in the measurement path, which should be ignored.

Figure 4. SuperAmine BRDF fitting of measured data and Zemax ABg models for simulation (a) BRDF in scatter angles and (b) BRDF in scatter vector magnitude.

We fit the measured BRDF characteristics of Super Amine slide with the ABg scatter model as shown in Fig. 6. Since the measured BSDF (red curve) of the slide is not a good fit with merely one ABg model as seen in Fig. 4(b), we used the combination of two ABg models (blue and green curves), and the summation of them (black curve) performed a very good fitting. We then defined an ABg model file to form the summation of the two ABg parameters (ABg model 1: g=1.8, B=0.016, A=0.0002 and ABg model 2: g=2.8, B=8e-6, A=3.6e-6) and imported the ABg file into Zemax, which efficiently supports a weighted combination of up to 20 ABg parametric sets for one specified scattering. Through our simulations, we found the scattering properties on the lenses and the mechanical holders have trivial impact on the final results, thus they can be set as no scatter or empirical values (e.g. lenses: g=2, B=0.015, A=0.003 and holders: Lambertian model with scatter fraction = 0.1).
Figure 5. Scatter modeling of the confocal fluorescence detection with slanted 60-degree illumination in Zemax (1 million rays with colors to indicate intensity, NA 0.79, pinhole diameter 200um) (a) Lambertian scattering model is adopted for ONCYTE and (b) ABg scattering model is selected for Super Amine.

After applying the above-mentioned scatter features, we have performed the non-sequential ray tracing in Zemax with 1 million rays. Figure 5 shows the scattering simulation result over the ONCYTE and Super Amine slides with 60-degree illumination. Note that the scatter yields are purely a percentage indicator to show how much scattered laser light can reach the detector without filtering which will be later considered in the SNR calculation. If the illumination is perpendicular, the simulation can be adjusted by changing the incident angle of the laser. Different numerical apertures (NAs) of the collection lens are simulated to see the change of scatter yield, and the results are summarized in Fig. 6. As we can see, the scatter yield of the SuperAmine slide has little change with increased NA, while the noise generated from the laser scattering on the ONCYTE slide increases fast with the NA change until it is larger than 0.6. The illumination angle has little impact over the ONCYTE slide due to its Lambertian alike BSDF distribution, while on the smooth SuperAmine slide, the illumination angles causes a big difference.

Table 1 describes an exemplary result when the NA is 0.4. For the ONCYTE slide with 60-degree tilt illumination, the scatter yield is about 40%; and a similar result is obtained for perpendicular illumination, in agreement with the uniformity of a Lambertian scatter. When a SuperAmine slide is used, we obtained a scatter yield of 0.02% with slanted 60-degree illumination, and 3.4% when the illumination angle is perpendicular. Note that the 60-degree slanted illumination on the Super Amine slide has the lowest laser scatter induced noise, about 2000 times lower than the ONCYTE slide in the same setup, and about 10 times lower than the perpendicular illumination using the same slide.

![Laser scatter yield change with different sample slides and numeric apertures](image)

Figure 6. Laser scatter yield change with different sample slides and numeric apertures: SuperAmine slide has constant unblocked laser noise with an increasing NA of the collection lens, while the ONCYTE slide typically has more laser induced noise with a larger NA.
3.3 Tolerance analysis with two different illumination paths

Alignment is not a trivial work in realizing an as-assembled system. We have analyzed the tolerance for the two optical configurations with perpendicular illumination and slanted illumination, as seen in Fig. 7. Perturbations due to misalignment or fabrication errors of the system are considered, which can be categorized as the defocus in z, decenter/tilt of x and y as well as the mirror position/tilt. The metric to quantify the performance is the collection efficiency loss, defined as signal loss on detector/nominal signal power*100%.

Figure 7. Tolerance analysis for (a) perpendicular illumination and (b) slanted illumination.

The tolerance analysis results are summarized in Table 2. Each perturbation is added to the system separately, then the obtained fluorescence signal is compared with the nominal signal to get the loss percentage. Within each category, we choose the max loss percentage to determine the sensitivity. One compensator is used when it can clearly improve the performance, otherwise it is specified as no compensator. From the results, we can see that perpendicular illumination ensures a much more robust system, all perturbation can be compensated with the pinhole position; while in the slanted illumination setting, the laser spot is difficult to be well aligned with both the conjugated pinhole and the sample. We also notice that, the slanted illumination gives an extended elliptical beam profile on the sample surface, which requires a larger field of view for the collection optical system.

Table 2. Comparison of the tolerance analysis results based on perpendicular and slanted illuminations

| Items | Perturbations | Perpendicular | Slanted |
|-------|---------------|---------------|---------|
|       |               | Compensator?  | Max loss | Compensator? | Max loss |
| Decenter/Tilt X and Y (Lenses, filters) | +/- 0.1mm or 0.1 degrees | Yes, pinhole X or Y | 1% | No | 5% |
| Defocus Z (Sample slide, spacers, thicknesses) | +/- 0.1mm | Yes, pinhole Z | 1% | No | 44% |
| Mirror Position/Tilt (X, Y, Z and Tilt X, Tilt Y) | X +/-0.1mm | No need | 1% | No | 67% |
|       | Z +/- 0.1mm | No need | 1% | No | 39% |
|       | Y +/- 0.1 degrees | Yes, pinhole X | 1% | No | 36% |
Simulation settings

Zemax non-sequential mode with 1% repeatability, laser source 10k rays, 5mW
*No compensator: no obvious improvement or need for more than one compensator

3.4 SNR simulation of a selected design

Although the slanted illumination gives a much lower scatter noise yield, we choose the perpendicular illumination on a Super Amine slide as it ensures moderate scatter noise with a robust alignment possibility. We aim for an extremely low abundance detection; therefore, the target molar concentration is set to be less than $10^{-15}$ molar in 1μL solution (<1pM/ml), the whole molecule abundance is much lower than state-of-the-art methods that use sample volumes around 100 μL to achieve a LOD of 0.1–1pM [2,4].

The other required parameters are collected and listed in Table 3. The laser diode spectrum is measured (Avantes VIS spectrometer). The spectra of the filter set (Semrock LED-Cy5-A) and the PMT quantum efficiency (QE) are provided by the supplier as plotted in Fig.8(a). Figure 8(b) shows the crucial spectra in logarithmic coordinates to see potential leaked laser noise as highlighted in black circles, if only the emission filter is used. The dark noise power is calculated from the PMT dark current equal to 0.6nA. Sample slides and other optical components will generate autofluorescence, which is another source of the generated noise. This value is usually obtained using the background noise measurements. For this simulation, we assume an empirical factor 0.01 as the ratio of the autofluorescence intensity to the signal intensity [29].

Table 3. Parameters for the SNR calculation

| Type No.   | Collect Parameters                                      |
|------------|--------------------------------------------------------|
| Laser      | Thorlabs CPS635F 5mW, CWL = 635nm, measured spectrum in Fig. 8(b) |
| Optics     | Objective Olympus NA0.4, 20x NA = 0.4, T_{opt1} = 0.9, T_{opt2} = 0.9 |
| Filters    | Semrock LED-Cy5-A FF01-635/18, FF01-680/42, FF652-Di01 spectra |
| Sample     | Alexa Fluor 647 DMAC = 270000, Quantum Yield = 0.33, normalized absorption spectrum and emission spectrum, molar concentration = 0.05, 0.1 and 0.5 pmol/ml, volume = 1μL |
| Holder     | Super Amine slide                                     |
| Detector   | Hamamatsu H-12400 Quantum efficiency = 10.3% (630nm), radiant sensitivity = 1.5x10^4A/W, dark current = 0.6nA |

Figure 8. (a) The corresponding spectra for SNR calculation (b) The spectra in logarithmic coordinates to clearly see the potential laser noise highlighted by the black circle
With all these parameters available, we calculated the SNR according to Eq. (7)-(15). The SNR is 6.8 when the molar concentration is 0.05 pmol/ml, as seen in Fig. 11. From the noise calculation, the dark current noise is the most influential factor in this design. Laser scatter noise can also have considerable impact without high optical density performance (e.g. OD > 6); however, high OD filters are expensive. We also notice that, even though the laser is supposed to have a very narrow FWHM (0.94 nm with this laser diode), the extremely weak power from non-lasing wavelengths can still destroy the super-sensitive detection if no excitation filter is used.

4. EXPERIMENTAL RESULTS

According to the SNR simulation and tolerance analysis, we built the setup as illustrated in Fig. 10. A blue LED is added to illuminate the sample for imaging on the CMOS camera, which avoids serious photobleaching during alignment. One more dichroic filter is used to separate the fluorescence signal towards the PMT and the blue image to the CMOS camera. Different concentrations of sample solutions were made by diluting with a corresponding volume of phosphate-buffered saline (PBS) to achieve concentrations of 0.05, 0.1 and 0.5 pmol/ml. The sample was made by pipetting 1 µl diluted dye solution, then dropped on a clean SuperAmine slide and dried under room temperature.

![Figure 10](image.png)

**Figure 10.** (a) The system design and (b) the experimental setup with pipetted sample spot on a SuperAmine slide

After the sample spots were made, we used the as-built demo setup to measure the SNR being the ratio of mean average signals to the standard deviation of the signals. 1000 data points were recorded for each measurement at 10k Hz, and three sample spots were measured for each concentration. Serious photobleaching makes the sample signal drops very fast, which results in a very short time for effective measurements. The measured data with error bar are plotted in Fig. 11. The experimental result shows a certain agreement, while the difference might lay in the evaluation error from auto-fluorescence or the different scatter between the dried sample and the laser.
5. CONCLUSION

We have established a modeling method that carefully considers the scatter induced laser noise and the generated fluorescence signal to determine the SNR for fluorescence detection. The method uses the ABg model or combined multiple ABg models as the scattering BSDF function for fitting realistic measured data, which provides an excellent match on typical optical surfaces. The exemplary simulations indicate that two sample slides (SuperAmine and ONCYTE) can have 10-times and 2000-times difference of scattered noise in perpendicular and in 60-degree slanted illumination respectively, so an effective modeling can help to identify the most contributing laser noise. The tolerance analysis for both perpendicular and slanted illumination shows that the perpendicular illumination has a more robust alignment, thus ensuring a higher feasibility. We built the perpendicular illumination setup based on tolerance analysis results and the experiments show an effective detection over 0.05 femtomole fluorescent molecules (a 1uL drop of sample with its concentration 0.05 pM/ml).

Note that the developed simulation tool is applicable for various confocal configurations (e.g. different illumination angles and filters), thus feasible to rapidly form a fair comparison among different options. This simulation tool therefore can be used to identify the dominant noise for further improving the LOD and to avoid over-functional, costly components (e.g. multiple filter set). Further work will focus on the identification of the influential factors that lead to difference of SNR simulation and experimental results.

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REFERENCES

[1] Schiffman, J. D., Fisher, P. G. & Gibbs, P. Early detection of cancer: past, present, and future. American Society of Clinical Oncology Educational Book. 35: 57-65 (2015).
[2] Nakamura, A. et al. High performance plasma amyloid-beta biomarkers for Alzheimer's disease. Nature. 554: 249-254 (2018).
[3] Li, Y. et al. Improving the limit of detection in portable luminescent assay readers through smart optical design. J. Biophotonics. 13: e201900241 (2020).
[4] Perez-Ruiz, E. et al. Digital ELISA for the quantification of attomolar concentrations of Alzheimer’s disease biomarker protein Tau in biological samples. *Anal. Chim. Acta*. **1015**: 74-81 (2018).

[5] Tan, Y., Sutanto, E., Alleyne, A. G. & Cunningham, B. T. Photonic crystal enhancement of a homogeneous fluorescent assay using submicron fluid channels fabricated by E-jet patterning. *J. Biophotonics*. **7**: 266-275 (2014).

[6] Cunningham, B. T. & Zangar, R. C. Photonic crystal enhanced fluorescence for early breast cancer biomarker detection. *J. Biophotonics*. **5**: 617-628 (2012).

[7] Santos, G. M. et al. Label-free, zeptomole cancer biomarker detection by surface-enhanced fluorescence on nanoporous gold disk plasmonic nanoparticles. *J. Biophotonics*. **8**: 855-863 (2015).

[8] Grilli, S. et al. Active accumulation of very diluted biomolecules by nano-dispensing for easy detection below the femtomorphic range. *Nat. Commun*. **5**: 5314 (2014).

[9] Ferraro, P. et al. Dispensing nano-pico droplets and liquid patterning by pyroelectrodynamic shooting. *Nat. Nanotechnol*. **5**: 429 (2010).

[10] Currie, L. A. Detection and quantification limits: origins and historical overview. *Anal. Chim. Acta*. **391**: 127-134 (1999).

[11] Pawley, J. *Handbook of biological confocal microscopy*. (Springer, 2006).

[12] Kurzbuch, D., Somers, M. & McDonagh, C. High efficiency ring-lens supercritical angle fluorescence (SAF) detection for optimum bioassay performance. *Opt. Express*. **21**: 22070-22075 (2013).

[13] Haider, S. A. et al. Fluorescence microscopy image noise reduction using a stochastically-connected random field model. *Sci. Rep*. **6**: 20640 (2016).

[14] Sharma, G. et al. Smartphone-based multimodal tethered capsule endoscopic platform for white-light, narrowband, and fluorescence/autofluorescence imaging. *J. Biophotonics*. **14**: e202000324 (2021).

[15] H. V. H. S. Axel Kasper, "Design considerations for highly effective fluorescence excitation and detection optical systems for molecular diagnostics," *Proc. SPIE* **1048**, 10486 (2018).

[16] Nie, Y. et al. "Optical aspects of a miniature fluorescence microscope for super-sensitive biomedical detection," in *Biophotonics Congress: Biomedical Optics 2020 (Translational, Microscopy, OCT, OTS, BRAIN)* (OSA, Washington, DC, 2020), pp. h3A-h4A17.

[17] Tanaka, K. et al. Compound parabolic concentrator probe for efficient light collection in spectroscopy of biological tissue. *Appl. Optics*. **35**: 758-763 (1996).

[18] Kim, J. K. et al. Fabrication and operation of GRIN probes for in vivo fluorescence cellular imaging of internal organs in small animals. *Nat. Protoc*. **7**: 1456 (2012).

[19] Venkata, S., Prasad, B. R., Nalla, R. K. & Singh, J. Scatter studies for visible emission line coronagraph on board ADITYA-L1 mission. *Journal of Astronomical Telescopes, Instruments and Systems*. 3: 014002 (2017).

[20] Stover, J. C. *Optical scattering: measurement and analysis; 3rd ed*. (SPIE press, Bellingham, 2012).

[21] Harvey, J. E., Choi, N., Schroeder, S. & Duparré, A. Total integrated scatter from surfaces with arbitrary roughness, correlation widths, and incident angles. *Opt. Eng*. **51**: 013402 (2012).

[22] Schröder, S. et al. Modeling of light scattering in different regimes of surface roughness. *Opt. Express*. **19**: 9820-9835 (2011).

[23] Zeidler, S. et al. Calculation method for light scattering caused by multilayer coated mirrors in gravitational wave detectors. *Opt. Express*. **25**: 4741-4760 (2017).

[24] Butler, S. D., Nauyoks, S. E. & Marciniak, M. A. Comparison of microfacet BRDF model to modified Beckmann-Kirchoff BRDF model for rough and smooth surfaces. *Opt. Express*. **23**: 29100-29112 (2015).

[25] Harvey, J. *Light-Scattering Characteristics Of Optical Surfaces*. *Proc. SPIE* **0107** (1977).

[26] Zemax Manual. (Zemax LLC, Kirkland, Washington, USA, http://www.zemax.com/, 2014).

[27] Pfisterer, R. N. Approximated scatter models for stray light analysis. *Optics & Photonics News*. **22**: 16-17 (2011).

[28] Lakwicz, J. R. *Principles of Fluorescence Spectroscopy*. (Springer, 2006).

[29] Anderson, N., Prabhath, P. & Erdogan, T. Spectral modelling in Fluorescence microscopy. Preprint at http://www.semrock.com/Data/Sites/1/semrockpdfs/spectral_modeling_in_fluorescence_microscopy.pdf (2011)

[30] Cuendet, M., Mesecar, A. D., DeWitt, D. L. & Pezzuto, J. M. An ELISA method to measure inhibition of the COX enzymes. *Nat. Protoc*. **1**: 1915 (2006).

[31] Kaushik, A. et al. Nano-biosensors to detect beta-amyloid for Alzheimer’s disease management. *Biosensors and bioelectronics*. **80**: 273-287 (2016).