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# Improving the sensitivity of protein microarray by evanescent-field-induced fluorescence

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**Abstract:** To improve the sensitivity of protein microarray, a prism surface replaces the surface of the common microscope slide. The protein targets arrayed on the surface are hybridized and labelled by fluorescent probes. Evanescent excitation occurs when the convergent laser reaches the surface, and a photomultiplier tube detects the emitted fluorescent signal. A two-dimensional actuator scans the whole surface to achieve planar laser excitation and fluorescence collection. The penetration depth of the evanescent field into the protein targets is only some hundred nanometers and can be controlled by different incident angle of the laser beam, so the undesired background signals are reduced dramatically and the detection sensitivity is improved by a factor of 50 to 100 comparing to confocal excitation. This approach can detect low abundance analytes without signal amplification.

**Key words:** Protein microarray, Evanescent excitation, Sensitivity, Total reflection
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## INTRODUCTION

The development of protein microarrays has accelerated within the past few years. Protein microarrays are widely used in diagnostics, drug screening and testing, disease monitoring, drug discovery and medical research. A protein microarray is a small surface (for example, a microscope slide) onto which the protein targets are immobilized, and hybridized and labelled with fluorescent probes. To analyze the microarray, a confocal microarray reader uses a laser to excite the fluorophores and a photomultiplier tube (PMT) then detects the resulting fluorescence, and the system produces an image that shows the intensity of the fluorophores (Wang *et al.*, 2004).

However, most of the cell proteins (up to 90%) are believed to be presented as low copy numbers (Miklos and Maleszka, 2001). Not like nucleic acid analysis, no biochemical target amplification scheme, like PCR, is available for proteins (Pawlak *et al.*, 2002), so it is very important in protein microarray platform to achieve the highest possible detection sensitivity.

In confocal microarray reader, the undesired background signals limit the detection sensitivity, which are mostly caused by the relatively long path of the excitation laser with high intensity before and after the surface where the protein targets are arrayed (Duveneck *et al.*, 2002). Typically, this path is four to six times larger than the beam waist. When the laser spot is 5 μm on the surface, this path will still be about 20 μm. At the same time, confocal scanning requires the flatness of the microarray slides within 10 μm, which is not satisfied by the specifications on thickness variations (50 μm) of standard microscope slides (Schena, 1999). If the sample is located outside the focus of the optical excitation and emission light path, the efficiency of fluorescence light collection and the excitation light intensity is strongly reduced. These thickness variations may correspond to unacceptably large measurement error (Rachlin, 2002).

The above problems can be avoided by evanescent field of excitation. A 100 nm to 200 nm thin film of a material with relatively high refractive index is deposited on the microarray slide with lower refrac-

tive index to form a planar waveguide. A parallel laser beam is coupled to the waveguiding film by a diffractive grating, etched into the microarray slide. The laser beam propagates within this film and creates a strong evanescent field. The field strength decays exponentially with the distance from the waveguide surface, and its penetration depth is limited to about 200 nm. This effect can be utilized to selectively excite only fluorophores located at or near the surface of the waveguide, resulting in a significant increase of the detection sensitivity (Duveneck *et al.*, 2002). But this method still has some problems. Firstly, sophisticated surface modifications must be applied to the thin film to stably immobilize the protein targets. This procedure is not compatible with the common microarray technology (Pawlak *et al.*, 2002). Secondly, the efficiency of the grating coupler is about 8%, so the excitation intensity is only 0.1~2 mW. Furthermore, it is difficult to emboss or etch the diffractive grating onto the microarray slide. Thirdly, the evanescent field is simultaneously on the area of the whole microarray slide, so the excitation density is relatively low and the fluorescent signal is weak. The CCD camera must be Peltier-cooled, increasing the cost of the system (Duveneck *et al.*, 2002).

In order to eliminate the above problems of planar waveguide technology, a novel protein microarray platform is introduced here. The protein targets are arrayed on a prism surface, and are then hybridized and labelled by fluorescent probes. Evanescent excitation occurs when the convergent laser reaches the prism surface at an angle greater than the critical angle, and a photomultiplier tube detects the emitted fluorescent signal. A two-dimensional actuator scans the whole surface to achieve planar laser excitation and fluorescence collection.

## PLATFORM DESIGN

The protein microarray platform is shown in Fig.1. After beam expander, the red laser beam (635 nm) is reflected by mirror 1, and then passes through the dichromatic mirror and reaches mirror 2. The dichromatic mirror is transparent in 635 nm and reflective in 532 nm, so the green laser beam (532 nm) after beam expander is reflected by it and reaches mirror 2. The optical paths of the two laser beams are the same after the dichromatic mirror. Mirror 2 directs the laser beams to pass through the pinhole in mirror 3 at a certain angle. Lens 1 congregates the laser beams and mirror 4 reflects them to the beam-in surface of the prism perpendicularly. The protein targets are arrayed on the prism surface, and the laser beams will be totally reflected when they reach the prism surface. The evanescent field is available at this point and excites the labelled fluorophores. A certain part of the emitted fluorescence will be reflected by mirror 4 to lens 1, which depends on the size of mirror 4 and numerical aperture (NA) of lens 1. After lens 1, a major part of the fluorescent beam will be reflected by mirror 3 to lens 2, while a minor part will be lost because of the pinhole in mirror 3. Lens 2 congregates the fluorescence beam into the photomultiplier tube (PMT). After signal amplifier and analog to digital (A/D) converter, the fluorescent signal is inputted into the computer. The fluorescent filters are located between mirror 3 and lens 2, and can be switched by a motor according the wavelength of the exciting laser. A linear actuator drives mirror 4 rapidly to scan the prism surface in one dimension to and fro at frequency of 20 Hz. A stepping motor moves the prism along the other dimension. So two-dimensional scanning is realized.

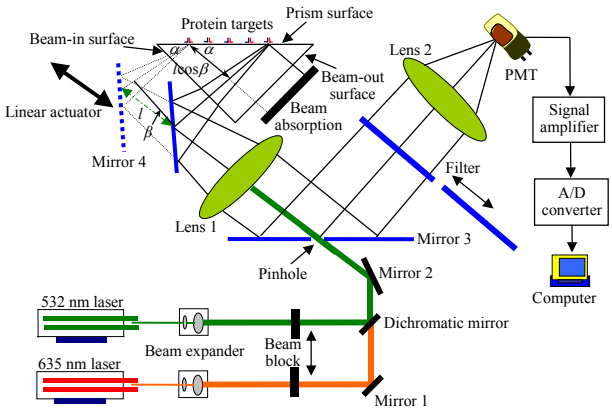


**Fig.1 Diagram of the designed protein microarray platform**

Given the linear actuator moves mirror 4 to two different points, the displacement between these two points is $l$ (Fig.1). The angle formed by the beam-in surface and prism surface is $\alpha$ and the angle formed by the beam-in surface and the optic ray axis is $\beta$. The optical length $x$ between the above two points is

$$x=nl\cos\beta\tan\alpha-(l+l\sin\beta) \quad (1)$$

$n$ is the refractive index of the prism. If $n$ is 1.5 and $\alpha$ is 45°, in order to ensure the optical length is zero between these two points, that is, $x$=0, the angle $\beta$ must be 22.6°. According to these parameters, the designed optical system can guarantee the same optical length at different scanning points and the same size of laser spot on the prism surface. Furthermore, both the excitation of protein targets and the collection of the fluorescence occur on the optic ray axis, so the optical design is rather simple.

Because the material of the prisms can be the same as that of standard microscope slides, the spotting procedure is compatible with the common microarray technology. As not all of the microarrays are reusable, the cost of the prisms is somewhat higher than that of microscope slides, although the standard microarray slide can also be used in the presented platform. After dropping a layer of liquid buffer on the prism and placing the microarray slide onto it, the prism can be reusable and the platform becomes more universal. Under this configuration, the refractive index of the liquid buffer matches that of the slide and the prism.

## SYSTEM PERFORMANCE

In the designed system, the maximal scanning width of the protein microarray is 20 mm, which means that the maximal optical length $l_f$ in the prism is

$$l_f=20n\sin\alpha=21.2 \text{ mm} \quad (2)$$

The focal length $f_1$ of lens 1 is 61.2 mm ($n$=1.5), so the minimal optical length outside the prism is 40 mm.

The optical resolution of the designed system is 10 μm, so the radius $r$ of the laser spot on the prism surface should be no more than 10 μm. Considering the diffraction of lens 1, the diameter $D$ of the laser beam ($\lambda$=635 nm) after expanding should be

$$D=1.22\lambda f_1/10\approx4.7 \text{ mm} \quad (3)$$

So the diameter $D$ is 5 mm in the presented system. The critical angle $\theta_c$ of total reflection on the prism surface is given by

$$\theta_c=\arcsin(1/n) \quad (4)$$

$\theta_c$ is 41.8° when $n$ is 1.5. The cone angle $\theta_l$ of the laser beam after lens 1 is given by

$$\theta_l=\arctan(D/2f_1)\approx2.3° \quad (5)$$

Because the principal ray of the laser beam is perpendicular to the beam-in surface of the prism, the incident angle $\theta_i$ of the principal ray on the prism surface equals $\alpha$, which is 45°. The minimal incident angle of the laser beam is $\theta_i-\theta_l$=42.7°, greater than the critical angle $\theta_c$, so all of the laser beam is total reflected, and all the fluorophores are excited by the evanescent field.

The numerical aperture of lens 1 is 0.4, so the collection efficiency is about 4.2 percent in the presented platform, somewhat lower than that in total internal reflection fluorescence microscopy (TIRFM).

In order to reduce the cross-talk error between Cy3 and Cy5 fluorophores, the prism surface is scanned sequentially, not simultaneously. That is, the 635 nm laser first scans the surface to obtain the Cy5 image, the 532 nm laser is blocked during this period and the fluorescent filter content corresponds to Cy5 dyes; and then the 532 nm laser scans the surface to obtain the Cy3 image, the 635 nm laser is now blocked instead and the filter content corresponds to Cy3 dyes. The fluorescent signal is detected by a photomultiplier tube and converted to electrical signal. After signal amplifier, the electrical signal is converted to digital numbers by a 16-bit analog to digital converter. Finally the fluorescent image of the microarray slide is reconstructed in the computer.

Although a Peltier cooled CCD camera offers greater speed than PMT in fluorescence detection, its dynamic range and sensitivity are not as good as those of PMT. At the same time, the Peltier cooled CCD camera is much more expensive than a high performance PMT. So a high sensitivity, low noise PMT is used to detect the emitted fluorescence light in the presented platform.

To ensure only emission photons from the appropriate fluorescent dyes reaching the PMT, the filter's window should match as closely as possible to the peak emission spectrum of the selected dyes, and maximize the desired emission light, and exclude stray laser light and other background light. The

transmission coefficient of the filter is above 0.8 within the entire specified band, and close to zero outside it.

A key character of the evanescent wave is that it propagates parallel to the interface, vanishing exponentially perpendicular to the interface. The penetration depth $z_m$ (the distance from the interface where intensity decreases to 1/e) of the evanescent wave along the depth of the field depends on the incident angle $\theta_i$, the wavelength of the excitation beam $\lambda$ and the diffractive indices of both media (Toomre and Manstein, 2001).

$$z_m = \lambda / \left[ 4\pi \sqrt{\left( n_1^2 \sin^2 \theta_i - n_2^2 \right)} \right] \quad (6)$$

here, $n_1$=1.5, $n_2$=1. When $\theta_i$=45°, $z_m \approx 0.5\lambda$; when $\theta_i$=60°, $z_m \approx 0.2\lambda$; when $\lambda$=635 nm, the penetration depth $z_m$ is about several hundred nanometers, 2 orders smaller than that of confocal excitation. Therefore, only surface-confined fluorescent dyes are selectively excited for emission, whereas excitation does not occur in the bulk medium. This spatial discrimination and the high excitation fields at the prism surface result in significant increase in signal-to-background ratios compared with conventional confocal optical detection methods. Some researchers (Duveneck *et al*., 2002) compared the fluorescent signals from one and the same microarray, excited by the evanescent field (Fig.2, left) and by the confocal laser beam (Fig.2, right).

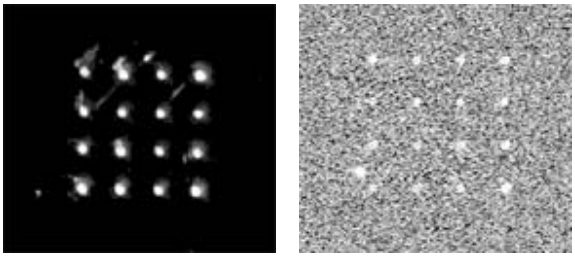

**Fig.2 Comparison of the result of evanescent field and confocal optics based excitation and detection using identical microarray**

Left: taken with the ZeptoREADER, an evanescent field based excitation scanner; Right: taken with a confocal fluorescence scanner

When the protein targets were excited by evanescent field, deviations from a perfect surface flatness in the range of more than 10~50 μm have no effect on the available excitation light intensities, as long as no light scattering is generated. Only the step of fluorescence collection is impaired by imperfections of the surface flatness, thus improving the tolerance on flatness variations.

## CONCLUSION

This paper presents a novel protein microarray platform to improve the detection sensitivity. A prism surface replaces the common microscope slide surface. The protein targets arrayed on the surface are hybridized and labelled by fluorescent probes. Evanescent excitation occurs when the convergent laser reaches the surface, and a photomultiplier tube detects the emitted fluorescent signal. A two-dimensional actuator scans the whole surface to achieve planar laser excitation and fluorescence collection. The penetration depth of the evanescent field into the protein targets is only some hundred nanometers, so undesired background signals are reduced dramatically and the detection sensitivity is improved by a factor of 50 to 100. This approach can detect low abundance analytes without signal amplification.

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