

Putting Photobleaching to Work

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Abstract

Protein microarrays’ ability to fluorescently detect low abundance molecules is limited by fluorescence noise emitted from surface chemistries and physical supports. Photobleaching of a sub-micron, protein-binding, nitrocellulose film on an opaque support selectively and permanently halves that noise. The effect on a number of protein binding commercial surface chemistry is presented at several energy levels of irradiation.

Introduction

Photobleaching of polymers is a well documented phenomenon. This paper shows how photobleaching can be used to minimize background fluorescence of protein attachment surface chemistry. Photobleaching can permanently halve the background fluorescence emitted by a sub-micron thick nitrocellulose film surface-chemistry and cause no biological degradation. The effectiveness of the process is shown to be strongly wavelength sensitive. Properties are tabulated for various commercial surface chemistries used for protein microarrays assays.

Photobleaching has historically been treated as the enemy by microscopy scientists. This remains true because the light making each sample visible also destroys its features; Becoming preeminent in fluorescence confocal microscopy where the local power level of the laser illuminated spot may exceed 5×10^5 Watt/cm² (1). The phenomenon is frequently referred to as a problem with no reliable method offered for protection. The natural background fluorescence of surface chemistry (*film or membrane*) and capture antibodies limit the detection and quantification of low abundance protein. The effect of the support (*typically a glass slide*) can be mitigated by the use of an opaque sub-layer (2) or a confocal scanner.

The Photophysical Problem of Photobleaching

Fluorescence is a phenomenon where the absorption of light by a fluorophore elevates electrons from the ground state to an excited state. Fluorescence light arises from the emission of a photon when the excited electron relaxes back to the ground state.

Photobleaching occurs when luminous flux interferes with or damages the fluorescence phenomena. This may be a temporary or permanent effect. Photobleaching in microscopy is reported by Tsien (1) and R. Mathies and L. Stryer (3) as a phenomenon that damages

biomolecules, especially dyes, used to emphasize features or identify properties of biomaterial. Photochemical damage is one of the most important and least understood aspects of fluorescence usage in biology. It is generally accepted that the magnitude of photobleaching is only a function of the accumulated energy imparted to the dye or polymer.

Photobleaching is often viewed as an aspect of photodegradation of polymers. This is presented as a generic effect which occurs through the activation of the polymer's macromolecules caused by the absorption of a photon of light.

In spite of extensive studies of photodegradation of polymers, there are many unsolved problems - Rabek (4). The process of nitrocellulose photodegradation has been studied by David Han and Tang Lie Gui (5) who illustrates measurable photodegradation taking place with exposure in excess of 1 hour of violet and UV light. Their report questions the association of photodegradation with oxidation (*as previously reported*) due to similar recorded degradation with samples in both air and vacuum.

Photobleaching of Surface Chemistry: Experimental Set-Up

Coated microscope slides, 25x75 mm, recommended for protein microarray assays were obtained from a number of vendors. Each slide was separated in two adjacent regions approximately 25x25 mm square. One region was enclosed in a light-tight shield and the other exposed. The slides tested were the HydroGel™ coated slides, the S&S membrane FAST™ slides and the DBI submicron nitrocellulose film PATH® slides.

The background signal level of HydroGel™ slide and a DBI micro film PATH® slide were recorded as received and found to exhibit similar background fluorescence level when scanned at 540 nm.

The S&S membrane FAST™ slides, prior to irradiation, exhibited background fluorescence 32.5 times higher than that of DBI PATH® slides when scanned at 540 nm.

The parameters of interest are the magnitude of fluorescence attenuation (*not the signal level*), stability and resulting biological activity. These parameters were documented as function of:

- Slide manufacturer
- Irradiation Energy level
- Irradiation wavelengths
- Imaging wavelengths
- Protein binding efficiency

Light Source - Illumination

Single high power Luminus LEDs were used for direct illumination. The LEDs had nominal center wavelengths at 450, 530 and 650 nm. Exposure duration was adjusted to define the total energy level of exposure.

A UV lamp illumination was also added.

The sun illumination is indicated only as a reference.

Power levels were measured with a “LaserCheck™” power meter from Coherent:

- UV : 10 mw/ cm²
- Green: 150 mw/ cm²
- Blue: 350 mw/ cm²
- Red: 160 mw/ cm²
- Sun radiation to earth @ 475 to 525 nm: 10 mw/cm²

Instrumentation

Fluorescence emission values were obtained by scanning the entire slide with a “PE ScanArray Gx™ microarray” confocal scanner (9). The Emission Ratios presented (*see Table: 1*) depict the ratio of average emission measured within 20 mm diameter circles. One circle is centered in the irradiated region, the other in the protected region. The parameters of the Scanner were adjusted for each class of slide to optimize measurement accuracy (i.e. to yield values between 3,000 and 30,000 arbitrary units).

A confocal microscope was used rather than a CCD/LED based imagers commonly used with protein microarrays in order to give a fair comparison between slides with (6) or without an opaque film blocking any fluorescence noise derived from the support. CCD/LED imagers have depth of field 10 times greater than those of the confocal microscope scanners.

Test Results

1 Effectiveness of Photobleaching

The table (*see Table: 1*) lists the measurements taken with three different surface chemistries. More data was taken when the process appeared to be more effective. The table lists the photobleaching wavelength, energy while reporting the emission ratio (post-irradiated emission divided by the pre-irradiated emission) at 530 and 633nm, without consideration of the pre-irradiation emission level. The data indicates that photobleaching is most effective on the sub-micron nitrocellulose film of DBI’s PATH® slides.

S&S FAST™ slides carry a membrane approximately 12 micron thick. That membrane is formed as a foam approximately half nitrocellulose and half micro-pores. The high level of signal detected is partly due to the fluorescence emission of the stimulated

nitrocellulose and partly by Lambertian re-emission of the excitation sources (7). This combination accounts for the 32-fold increase in the background signal of the S&S

surface chemistry compared to the thin film emission. Photobleaching only affects the nitrocellulose contribution and consequently is not an attractive process to lower background signal.

The HyperGel™ coating is approximately 4 micron thick as compared to the ½ micron of the PATH® slides. The attenuation ratio of the two slides is similar to their thickness ratio.

It is interesting to note that:

- Photobleaching is wavelength sensitive and alters fluorescence emission at or near the irradiation wavelength.
- Photobleaching is a function of the mass of the material affected and consequently most effective on film surface chemistry.

2- Stability of Photobleaching

Figure 1 shows that in the case of sub micron nitrocellulose film coating, recovery from photobleaching induced background noise attenuation is moderate: about 10% and takes place within a month following irradiation.

3- Biological Activity

Biological activity was studied only on the surface chemistry most affected by radiation. Four PATH® slides were selected: slides exposed @ $\lambda=450$ nm with 168 and 252 joule/cm² and slides exposed @ $\lambda=530$ nm with 77 and 108 joule/cm².

Each region was spotted with 4 arrays representing 8 cytokines. All slides were processed as per the standard protocol used at DBI and scanned with the PE ScanArray Gx™ microarray scanner.

In all cases no statistically meaningful molecular binding difference could be detected between the irradiated and protected regions.

Conclusion

Photobleaching is an effective treatment to permanently lower the natural fluorescence of most polymer-based surface chemistries used for protein microarray work. This photobleaching does not alter the biological activity of a nitrocellulose surface.

Photobleaching is cost effective only where the polymer coating is extremely thin. Where the polymer surface chemistry is a sub micron thickness of nitrocellulose, natural fluorescence can be economically cut in half with commercial LED sources. This benefit is wavelength selective and most advantageously used in the blue/green region of the spectrum.

Additional studies should be undertaken to evaluate the practicality of suitably filtered sun exposure instead of LEDs. The sun radiations listed above in the blue-green region indicates that photobleaching could be achieved with a few days exposure.

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References

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- (6) PATH™ slides, Fisher Scientific & GenTel Bioscience.
- (7) J. Montagu US patent 7,199,360
- (8) HydroGel™ -Coated slides from PerkinElmer Life Sciences as a Substrate for Multiplexed Immunoassays in a Microarray Format
- (9) "PE ScanArray Gx™ microarray; Referring to PerkinElmer's confocal scanner.

Figure 1-Photobleaching Stability: Emission level versus time and irradiation intensity

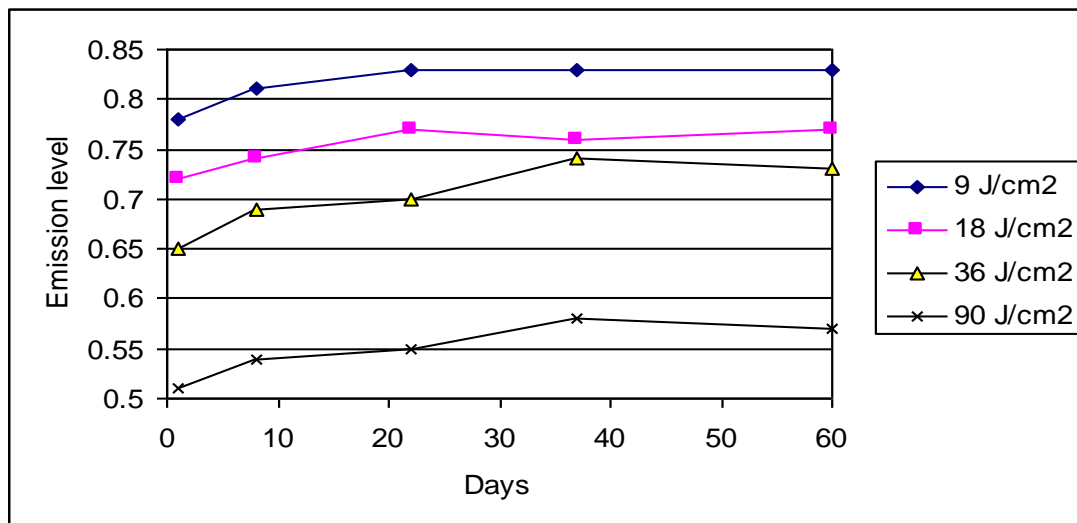


Table 1 –Slide Function: Fluorescence emission or coated slide as a function of wavelength and energy irradiation as well as wavelength detection.

DBI - PATH[®] Slide

<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>UV (350 nm)</i>	<i>@ 530 nm</i>	
2.4 joule/cm2	1.01	
<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Blue (450 nm)</i>	<i>@ 530 nm</i>	<i>@633 nm</i>
42 joule/cm2	.84	
84 joule/cm2	.67	1.01
168 joule/cm2	.53	
252 joule/cm2	.46	
<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Green (530 nm)</i>	<i>@ 530 nm</i>	<i>@633</i>
36 joule/cm2	.67	
72 joule/cm2	.53	
108 joule/cm2	.45	
216 joule/cm2	.38	.86
<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Red (636 nm)</i>	<i>@ 530 nm</i>	<i>@633 nm</i>
38 joule/cm2	.93	.80
115 joule/cm2	1.09	.87

S&S FAST[™] Slide

<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Blue (450 nm)</i>	<i>@ 530</i>	<i>@ 633</i>
42 joule/cm2	.96	1.02
168 joule/cm2	.84	1.01
<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Green (530 nm)</i>	<i>@ 530</i>	<i>@633</i>
36 joule/cm2	.81	1.04
108 joule/cm2	.70	1.02

HydroGel[™] Slide

<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Blue (45 nm)</i>	<i>@ 530</i>	<i>@ 633</i>
42 joule/cm2	1.06	1.05
168 joule/cm2	.65	
<u>Irradiation</u>	<u>Emission Ratio</u>	
<i>Green (530 nm)</i>	<i>@530</i>	<i>@633</i>
36 joule/cm2	.91	.98
108 joule/cm2	.79	

