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A whole new way of looking at things: The use of Dark Reader technology to detect fluorophors

The Dark Reader™ optical system uses relatively low intensity broad-band visible blue light in combination with broad-band optical filters to detect fluorescence with a level of sensitivity that often surpasses that of UV transilluminators and can rival that of laser-based scanners. Applications of DR™ devices include the detection of SYBR®-stained nucleic acids and SYPRO®-stained protein samples following, and also during, electrophoresis. Unlike laser-based imaging systems, the fluorescence is directly visible to the user as well as being fully compatible with CCD and Polaroid camera-based detection and imaging. Additionally, the DR optical system functions well in multi-color fluorophor environments. Because the Dark Reader does not emit any UV light, the extent of DNA damage incurred when visualizing DNA samples is drastically reduced compared to the damage produced by a UV device and this can have a significant benefit on downstream cloning protocols. Furthermore, dye photobleaching is minimal, extending the length of time that a fluorescent sample is visible. The inherent flexibility of the DR optical system allows many different configurations of the Dark Reader to be constructed such as transilluminators, hand lamps and integrated transilluminator-electrophoresis units.

Keywords: Dark Reader, fluorescence, DNA damage, imaging, proteomics, SYBR

1 Introduction

The enhanced fluorescence of ethidium bromide upon binding to nucleic acids was first exploited for the detection of DNA in gels following electrophoresis by Sharp et al. in 1973 [1]. Since then, this technique has become perhaps the most ubiquitous technique used in Molecular Biology. Unfortunately, not only is ethidium bromide a powerful mutagen but visualization of the fluorescence patterns of DNA in electrophoresis gels typically requires the use of UV light which is potentially hazardous.

In spite of draw-backs of this kind, fluorescence-based detection systems are, in general, very attractive because of their simplicity, speed, quantitative character and sensitivity of detection. Consequently, in recent years more and more fluorescence detection-based methods have been added to the repertoire of Molecular Biology techniques. Some of these methods rely on standard fluorophors such as fluorescein and rhodamine but much of the growth in fluorescence-based detection technology derives from the introduction of newly developed fluorophors. For example, several novel fluorescent stains for nucleic acids [2-5] and proteins [6, 7, 8] have been recently developed, as well as new enzyme-linked substrates such as AttoPhos™ (Promega, Madison, WI, USA) [9, 10] and DDAO-phosphate [11], covalent labels such as the BODIPY (Molecular Probes, Eugene, OR, USA) [12] and Cy (Amersham Pharmacia Biotech, Uppsala, Sweden) [13] series of fluorophors, and intrinsically fluorescent proteins such as GFP variants [14-16] and protein-chromophore complexes such as PBXL (Marktek Biosciences, Columbia, MD, USA) [17].

Along with the increasing use of fluorescence techniques, the current explosive growths in the fields of genomics [18] and proteomics [19, 20] is presenting ever-increasing demands on optical technology to deliver the sensitive, rapid and economical detection and imaging of fluorophor patterns in 2-dimensions such as gels, blots and even in 3-dimensions such as whole plants and animals.

1.1 Visualizing fluorescence

Typically, the direct visual detection of fluorescent species dispersed in gels and other media has required the use of a UV transilluminator as the source of excitation. The use of UV light is not particularly appropriate as, in fact, many

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Abbreviations: EtBr, ethidium bromide; FL, fluorescein; GFP, green fluorescent protein; TMR, tetramethylrhodamine

fluorophors used in the biosciences are more effectively excited by visible light. Figure 1 shows the excitation spectra of several popular fluorophors from the UV region through the visible. It is clear from a consideration of the excitation profiles that most of these fluorophors are excited to a significantly greater extent in the visible region than in the UV.

Until recently the only instruments available for the detection of fluorophor patterns that used visible light excitation were scanning laser- or light-emitting diode (LED)-based systems combined with photomultiplier-based detection such as the FMBIOII from Hitachi Genetic Systems (Alameda, CA, USA) and the Storm 840 from Molecular Dynamics (Sunnyvale, CA, USA). Such instrumentation has several disadvantages, the main one being that a fluorescent sample cannot be directly viewed by the naked eye. Consequently, it is not possible to examine the pattern of fluorescence in a gel directly or cut out bands from the gel. Other disadvantages of laser scanning-based detection instruments include the long acquisition times required (typically around 5 minutes is required for a typical mini-gel). The devices are also restricted to imaging samples close to the focal plane and cannot be used with three-dimensional samples or specimens. In addition, the complex scanners are prohibitively expensive for many laboratories. Also of note is the fact that the efficiency of a laser excitation is constrained by the very wavelength precision that is the hall-mark of these devices: because laser light has intrinsically a very narrow band-width of just a few nanometers, a laser light source is unable to excite a fluorophor over the entire wavelength range of the excitation spectrum which typically covers 100 nm or more.

Fluorescence spectroscopy, which is routinely used to quantitate fluorescence intensities in solution, typically employs instrumentation equipped with high intensity very broad-band visible light (‘white light’) sources for the excitation of fluorophors in combination with either narrow band-pass filters or monochromators for wavelength selection and photomultipliers for detection. Fluorometers, of course, can only be used to measure the fluorescence intensities of homogenous solutions and are of no use for recording images of fluorophor patterns in gels. In an approach taken from the basic principles of fluorometry, an imager can be constructed from a high intensity (~150 W) xenon arc source combined with a scanning CCD camera as, for example, in the Arthur™ 1442 Multi-Wavelength Fluorimager (PerkinElmer, Inc.). Both the lamp and detector are equipped with an inter-changeable selection of narrow band-pass filters. The use of narrow band-pass filters is necessary to reduce the ‘leakage’ of light from the light source that would result in an unacceptably high background signal from the powerful lamp and hence reduce the detectable fluorescence signal. Typically, narrow band pass filter transmit light only over a region of ~10 - 15 nm. The inefficiency of this type of optical configuration is reminiscent of that found in laser-based systems and indeed, powerful visible light source imaging suffers from many of the same drawback as laser-based scanning: the narrow wavelength selection reduces the amount of useful light, multiple scans are required to image multiple dyes, the devices are complex, cumbersome and expensive and it is not possible for the user to directly view the gel.

A somewhat less complicated approach described recently [21] utilized 2 x 100 W halogen lamps and a fixed CCD camera, all equipped with the appropriate narrow band-pass filters. Unfortunately, this device proved very inefficient and required 30 minute exposure times to achieve the highest levels of detection. Clearly, such a time-scale for data acquisition is impractical for routine gel documentation, kinetics experiments, delicate samples, or if the sample moves (e. g., live organisms). Furthermore, the device cannot be used to effectively view fluorescence emission patterns by the naked eye.

Because of these drawbacks, the UV irradiation of fluorescent samples, in the form of a simple UV transilluminator or UV hand lamp, has been the only practical alternative for many laboratories for the visualization and imaging of fluorophor patterns in electrophoresis gels. This approach has the advantage that samples can be seen directly, and when used in combination with a CCD or Polaroid camera, provides a reasonable level of sensitivity of detection. The major drawback, of course, is the potentially harmful nature of UV light. This danger has been largely ignored but can potentially have a deleterious impact on both the user and the integrity of biological samples.

1.2 A new approach

A unique approach has been taken recently to the problem of imaging fluorescent patterns that is based on a consideration of the entire wavelength ranges of the lamp and fluorophor spectra rather than narrowly-defined excitation and emission maxima. This approach resulted in the development of an intrinsically safe, low-intensity optical system that utilizes the maximum possible wavelength regions of both the light source and the excitation and emission spectra of fluorescent dyes to achieve the maximum fluorescent signal while simultaneously minimizing the background light caused by

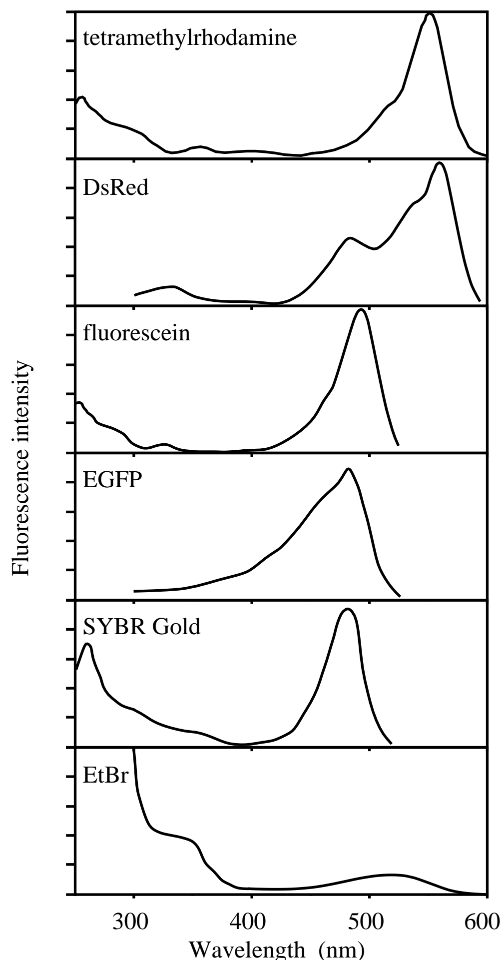

Figure 1. Fluorescence excitation spectra from the UV into the visible wavelengths for a number of popular and widely used fluorophors. For many fluorophors, the major excitation region is in the visible wavelengths between 400 and 500 nm. Even many of the fluorophors with excitation maxima in the red still exhibit substantial excitation between 400 and 500 nm. The spectra should be compared with the lamp output spectra in Fig. 2.

the exciting light ‘leaking’ through to the viewer. In a simple, single configuration, this approach is applicable to a broad range of fluorophors, even those with small Stokes’ shifts, and provides a very high level of sensitivity of detection both by the naked eye as well as CCD or Polaroid camera imaging systems. The optical system is referred to as Dark Reader™.

2 How Dark Reader technology works

The ubiquity of UV transilluminators in Molecular Biology laboratories for the direct visualization of fluorescent gels, and the lack of any practical alternative, has caused many researchers to forget the fact that the excitation maxima for many popular fluorophors are in the visible region of the spectrum, not in the UV (Figure 1). The Dark Reader optical system is specifically designed for such ‘visible region’ fluorophors.

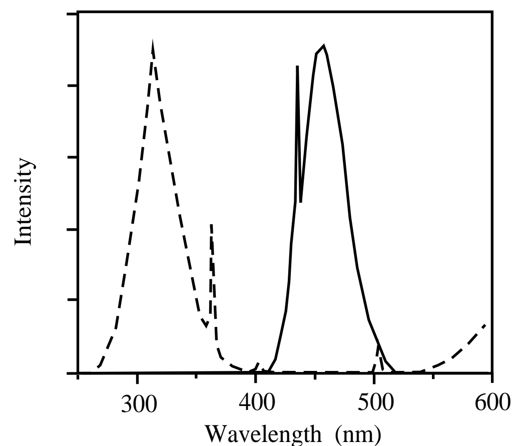

Figure 2. Lamp output intensity spectra for UV and DR lamps. The spectra should be compared with the fluorescence excitation spectra in Figure 1.

2.1 Optical components

As shown in Figure 2, the lamps used in Dark Reader devices generate maximum light output over a fairly broad range between 400 and 500 nm - close to where many popular dyes such as SYBR Green, SYPRO Orange, red-shifted GFPs, and fluorescein are maximally excited. UV transilluminators, on the other hand, typically output light around 300 nm, well removed from the absorption maxima of many common dyes.

If one attempts to a view fluorescent sample using a visible light excitation source alone, the fluorescence is barely visible to the naked eye due to the large amount of light from the light source itself that reaches the observer and effectively swamps the intrinsically very low intensity fluorescence emission. The Dark Reader transilluminators and other DR devices achieve the removal of lamp light in 2 steps (Figure 3). A broad-band blue filter is situated between the excitation light source and the fluorophor sample. This filter absorbs any residual green and red components emitted by the lamp and allows through to the sample only blue excitation light. Depending on the DR device, the blue filter can also act as the transilluminator surface or electrophoresis gel bed. The ability of the optical filters to act as integral structural components of DR devices is a natural consequence of the material from which the filters are manufactured - plastic - and consequently there are few, if any, constraints on the size, shape or design of the types of DR devices that can be constructed. This situation is in contrast to the small, delicate and expensive band-pass filters used in conventional visible-light imaging instrumentation.

A second optical filter is placed between the sample and the observer. This long-pass, amber filter effectively

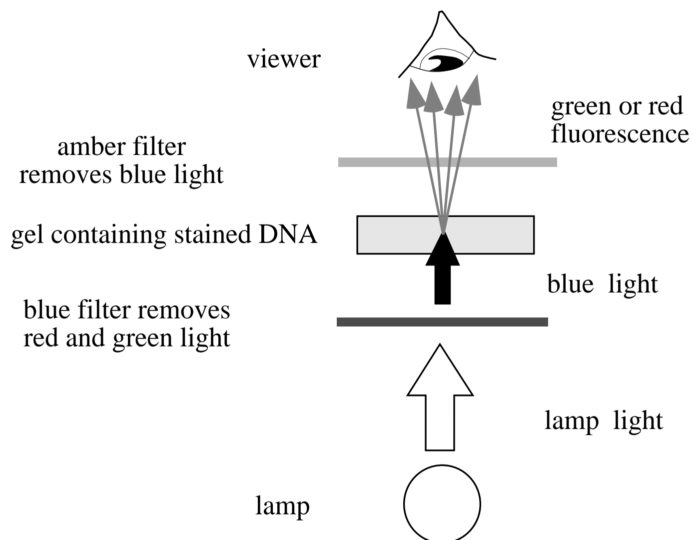

Figure 3. A summary of the basic optical configuration used in DR devices.

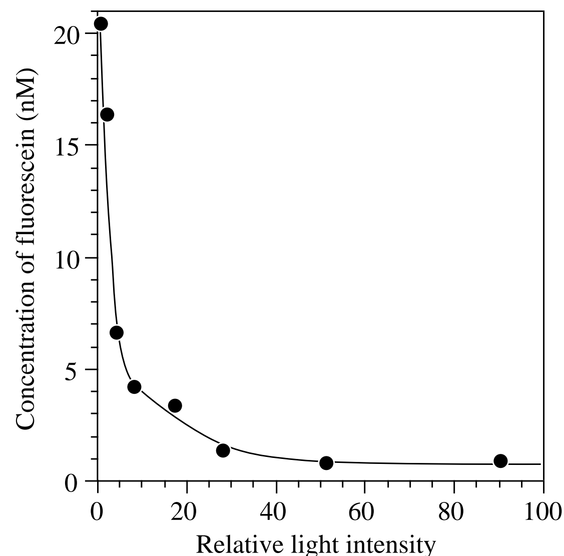

Figure 4. The detectability of fluorescence by eye as a function of the intensity of the excitation light. A set of 100 uL samples of a serial dilution of fluorescein (50 nM - 0.3 nM) in 200 uL plastic tubes were distributed on the surface of a DR45 transilluminator in which the intensity of the excitation light was varied by the use of attenuating screens, and examined by eye. A set of tubes containing buffer only was also mixed in among the fluorescent samples. At each light intensity those tubes containing detectable fluorescence were selected and documented.

removes the blue lamp light but allows passage of almost all the red and green fluorescent components from the sample allowing the sample to be clearly visualized. As with the blue filter, the amber filter may be configured to just about any design. Versions of the second filter include the glasses worn by the viewer, a simple screen that covers the top of a transilluminator and the safety lid on electrophoresis-transilluminator units.

Because of the high efficiency of the DR filter system in transmitting useful light and blocking spurious background light, a high intensity light source is not a required component in the DR optical system and most Dark Reader devices are based on a 9 W lamp. This is at least 10 times less powerful than the lamps that are necessary in narrow band-pass filter systems to compensate for the intrinsic inefficiency of this type of filter which block a large percentage of both the usable excitation and emission light.

In general, the intensity of fluorescence emission is directly proportional to the intensity of the excitation light and a first analysis might suggest that the use of a more powerful excitation source in Dark Reader devices would result in a corresponding increase in detectable fluorescence from samples. In fact, a plot of the detection limit of a fluorophor by eye versus light intensity is distinctly biphasic. (Fig. 4). At lower excitation light levels the fluorescence emission is too feeble to be detected by the human eye except in the most concentrated samples. As the excitation intensity is increased, the fluorescence emission attains a sufficient level to register on the retina and the detection limit increases rapidly as a function of excitation intensity. As the intensity is increased further, however, the leakage of excitation light through the filter system becomes significant. In this situation it is necessary for the eye to distinguish fluorescence from a visible background. Consequently, the detection limit becomes relatively insensitive to further increases in light intensity. Indeed, it can be predicted that at yet higher light levels the detection limit will actually be worse as the eye will have to distinguish a small difference between two relatively high light levels. The background leakage cannot be reduced by, for example, increasing the filter density as any subsequent reduction in background also results in a corresponding reduction in either excitation or emission intensity.

A camera, of course, does not have the same limitations as the human eye. However, there are still several practical restrictions to consider: when using low intensity excitation light, recording the correspondingly weak fluorescence can require an inordinately long integration time and the accumulation of excessive noise in the image. With high intensity excitation light, the situation in analogous to that with the human eye and it becomes necessary to distinguish actual fluorescence from relatively intense background leakage.

The use of a blue phosphor in the DR lamp with maximum output concentrated in the wavelength region between 400 - 500 nm is a key feature of the Dark Reader optical design: not only is the maximum output aligned with the excitation spectra of many fluorophors, but the intensity of the lamp

in the fluorescence emission region (above 500 nm) is very low, minimizing the potential background. The efficiency of the blue lamp is illustrated by a simple comparison: a 400 W (‘white light’) halogen lamp excitation source, even when equipped with appropriate broad-band filters, is 5 to 15-fold less sensitive than the Dark Reader configuration for the detection of fluorescein and tetramethylrhodamine, and when equipped with narrow band-pass filters the sensitivity of detection using the halogen lamp is at least 25-fold lower (unpublished results).

In summary, until the development of the Dark Reader optical system, the conventional wisdom has been that the most sensitive visualization or imaging of a fluorophor required an optical configuration in which a high intensity excitation light is tightly restricted to the excitation wavelength of the fluorophor by a narrow band-pass filter and the fluorescence detector is, likewise, constrained by a narrow band-pass filter chosen for its alignment with the emission maximum of the particular fluorophor. In fact, this type of optical configuration eliminates much of the useful excitation and emission light, not only lowering the sensitivity of detection but also making the instrument highly ‘fluorophor specific’ unless the specific filters are changed accordingly.

The design of the Dark Reader optical system goes against much of the generally accepted principles of fluorescence imaging in its use of broad-band / long-pass filters that exploit the maximum possible regions of both the excitation and emission spectra of a fluorophor. In combination with a relatively low intensity lamp with output concentrated in the blue, the Dark Reader optical system provides a sensitivity of fluorophor detection that, in many cases, often surpasses that of UV transilluminators and is equal to that of laser-based systems.

2.2 Imaging a broad range of fluorophors

Dark Reader technology can be used to detect many different fluorophors. In general, the ideal spectral characteristics for a ‘DR-dye’ are an excitation maximum between 420 - 500 nm and an emission maximum above 520 nm. It should be emphasized, though, that the Dark Reader can also be effectively used to detect dyes with maxima outside the above ranges. The only criteria for viewing a fluorophor are that at least a portion of the fluorescence excitation spectrum is between 420 - 500 nm and a portion of the emission spectrum is over 520 nm. Stated another way, Dark Reader devices can be used to detect almost any dye excited in the visible range that does not emit exclusively in the blue. Table 1 lists a few commonly used dyes and their viewability using the Dark Reader.

Table 1. Common fluorophors and their compatibility with the Dark Reader optical system

Dye	Ex/Em Maxima (nm)	Viewability
acridine orange	500/526	+ + +
aminoacridone	425/531	+ + +
AttoPhos®	440/560	+ + +
ATTO-TAG™	486/591	+ + +
BODIPY® FL	502/510	+ +
Cy3	552/568	+ +
DDAO	478/628	+ + +
DsRed	558/583	+ + +
EBFP	380/440	+
ECFP	434/477	+ +
EGFP	488/507	+ + +
eosin	524/544	+ +
Ethidium bromide	518/605	+ +
EYFP	513/527	+ + +
fluorescamine	381/470	-
fluorescein	492/525	+ + +
GelStar®	493/527	+ + +
Hoescht 33258	350/460	-
lucifer yellow	428/533	+ + +
NanoOrange™	485/590	+ + +
NBD	465/535	+ + +
OliGreen™	498/518	+ + +
PicoGreen®	502/523	+ + +
PyMPO	415/570	+ + +
SYBR® Gold	495/537	+ + +
SYBR Green I	494/521	+ + +
SYPRO® Orange	470/570	+ + +
SYPRO Ruby	450/610	+ + +
SYPRO Tangerine	490/640	+ + +
Tetramethylrhodamine	555/580	+ +
Vistra® Green	497/520	+ + +

+++, highly sensitive; ++, sensitive; +, can be used in some applications; -, not compatible; DDAO, 1,3-dichloro-9,9-dimethylacridin-2-one-7-yl, DsRed, a Red Fluorescent Protein from *Discosoma striata*; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; NBD, nitrobez-2-oxa-1,3-diazole derivatives.

It should be emphasized that the excitation and emission maxima provide only a rough guide to the utility of the various dyes with Dark Reader devices and complete spectral profiles should be studied to determine suitability.

Table 2. A comparison of the sensitivities of detection by eye for 2 common fluorophors using Dark Reader or 312 nm UV devices.

Dye environment	Ratio of minimum detectable amount of dye (DR / UV)	
	Fluorescein	Tetramethyl-rhodamine
plastic wrap (*trans*)	2	0.5
nitrocellulose membrane (*epi*)	2	2
vertical electrophoresis glass plate (*trans*)	4	4
96-well plate (*trans*)	8	4
1.5 mL polypropylene tubes (*trans*)	8	2
nitrocellulose membrane (*trans*)	16	8
horizontal electrophoresis acrylic (*trans*)	16	16

2-fold dilution series of fluorescein (FL) and tetramethylrhodamine (TMR) were variously aliquoted onto several media or laboratory-ware including plastic wrap, nitrocellulose membrane, 1 mm thick vertical electrophoresis glass plate, 6 mm thick horizontal gel apparatus clear acrylic, 96-well plate and 1.5 mL polypropylene tubes. The dilution series were then viewed by eye in either a '*trans*' configuration on a DR transilluminator or a 312 nm UV transilluminator or in an '*epi*' configuration using either a DR hand lamp or a 312 nm UV handlamp to illuminate from above, and the lowest detectable amounts of FL and TMR were recorded. For the sake of clarity, the results in the table are presented as the ratio of the lowest detectable amount of fluorophor using a DR device versus that using a UV device. The actual volumes used and the concentrations representing the minimum detection limits using the DR devices were as follows: plastic wrap - 1 uL, 62 nM FL, 312 nM TMR; nitrocellulose (epi) - 0.5 µL, 62 nM FL, 39 nM TMR; nitrocellulose (trans) - 0.5 µL, 125 nM FL, 156 nM TMR; glass plate - 2 µL, 62 nM FL, 312 nM TMR; 96-well plate -100 µL, 16 nM FL, 39 nM TMR; 1.5 mL tubes - 400µL, 4 nM FL, 10 nM TMR; acrylic sheet - 2µL, 62 nM FL, 312 nM TMR.

2.3 Detection of fluorophors in the laboratory environment

The handling and viewing of fluorescent dyes in the laboratory typically involves numerous types of media and containers including electrophoresis apparatuses, blotting membranes, test-tubes, etc. Consequently, the practical limits of fluorescence detection depend on a variety of factors besides the intrinsic optical properties of the dye and the viewing device. These factors include attenuation of the fluorescence excitation and emission as well as background fluorescence from the medium or container.

The experimental data summarized in Table 2 compare the sensitivity of DR and UV devices for the direct visual detection of fluorescein (FL, ex / em maxima = 492 / 525 nm) and tetramethylrhodamine (TMR, ex / em maxima = 555 / 580 nm) in a variety of typical laboratory settings.

The Stokes' shifts of both FL and TMR are relatively small (~25 nm) - a situation that conventional wisdom would suggest is not amenable to the use of the broad-band / long-pass visible light used in the Dark Reader. Furthermore, the TMR excitation maximum lies well outside the output range of the DR blue lamp and consequently it would be predicted, based on the excitation maximum alone, that use of a DR device would result in a poor fluorescence signal. The collected data show, however, that the performance of the Dark Reader optical system with both FL and TMR is, in many instances, significantly better than that of a UV device. This is directly attributable to the blue excitation band employed by the DR optical system which efficiently excites the fluorophors - even TMR (which does, in fact, have a significant blue component to its excitation spectrum). In addition, the blue exciting light of DR devices is not significantly blocked by plastic or glass, whereas 300 nm UV light does not penetrate well through such materials.

In a second measure of the performance of the DR optical system a comparison was carried out between a DR transilluminator, in combination with a CCD camera, and a visible laser scanner with photomultiplier detection. The results of a study on the ability of the two devices to

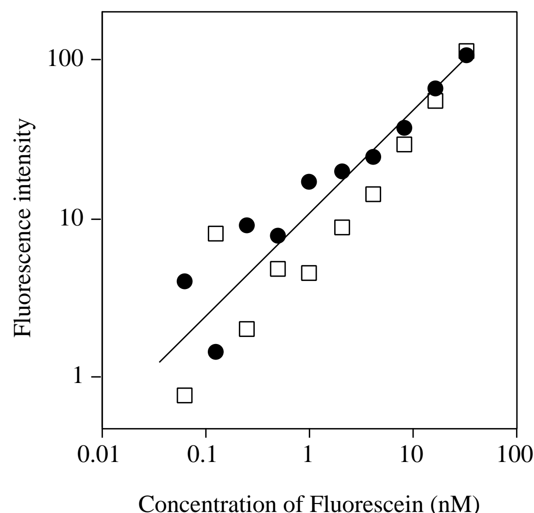

Figure 5. A comparison of the detection limit of a DR device and a laser scanner. 100 µL samples of a serial dilution of fluorescein were placed in a 96-well plate and imaged using either (●) a DR45 transilluminator in combination with a CCD camera, or (□) a laser scanner (FMBIOII, Hitachi Genetic Systems). The DR transilluminator performs as well as the laser scanner and can be used to reliably detect fluorescein down to a concentration of less than 1 nM (0.1 pmoles).

detect fluorescein emission is shown in Figure 5. The limit of detection with both devices is very similar at about 0.1 nM. The ability of the broad-band DR transilluminator to detect comparable levels of fluorophor as the extremely narrow-band laser-based instrumentation illustrates the inherent efficiency of the broad-band optical system used in the Dark Reader.

2.4 Viewing and imaging multiple fluorophors simultaneously

The importance of multi-color fluorescence detection will increase significantly in the future as the demands of large-scale projects in genomics, proteomics and drug-screening seek to maximize through-put. The broad wavelength range covered by the Dark Reader optical system permits the visualization and discrimination of multiple fluorophors in a single image without the need to change filters. In contrast, when instrumentation using either CCD cameras or photomultiplier tubes equipped with narrow band filters, it is necessary to either acquire and then process multiple images or to increase the number of light sources or detectors in the instrument to achieve this goal.

The ability of the Dark Reader optical system to detect and distinguish multiple fluorophors simultaneously was tested using a pair of SYBR Green- and SYBR Gold-stained DNA electrophoresis gels. The emission maxima of these dyes are 521 and 537 nm respectively - a separation of just 16 nm. Images of the 2 gels were recorded side-by-side on a DR transilluminator using a 'consumer-grade' color CCD-based digital camera (Olympus, Inc.). The fluorescence intensities recorded in the red and green channels of the color images were then plotted separately. Figure 6 shows the clear difference in the relative intensities of the red and green channels for the 2 fluorophors. The SYBR Gold-stained DNA exhibits a higher red channel intensity than does SYBR Green, as would be predicted based on the slightly red-shifted emission spectrum of this dye.

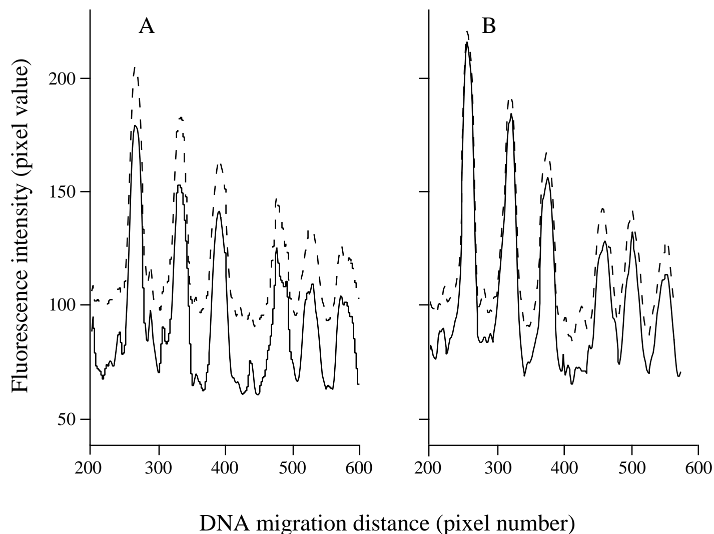

Figure 6. An illustration of the utility of the DR optical system for the imaging of patterns containing multiple fluorophors. A SYBR Green-stained gel (A) and a SYBR Gold-stained gel (B) of lambda DNA cut with SauI/StyI were photographed side-by side on a DR transilluminator using a color CCD camera (Olympus, Inc.). The pixel values in the individual red (solid line) and green (dashed line) channels of the color images were plotted for a sub-section of the gels containing several DNA bands. The red channel intensity is significantly higher in the Gold-stained gel (emission maximum ~537 nm) than in the Green-stained gel (emission maximum ~521 nm).

3 The harmful effect of radiation

Many users of standard UV transilluminators have experienced, at one time or another, either a mild case of sun-burn or 'spots before the eyes' as a result of spending too long either examining a gel or cutting out bands and most 'cloning manuals' warn of these dangers. The potentially harmful effects of shorter wavelength light are well documented [22-25] and were the subject of a report from the Council on Scientific Affairs of the American Medical Association [26]. High-intensity UV radiation can cause erythema, degenerative and neoplastic changes in the skin, retinal damage and cataracts, and modification of the immunologic system of the skin. Even the fluorescent lamps commonly used in homes and businesses emit sufficient UV light to cause mutagenesis in Salmonella [27] upon prolonged exposure. This latter effect is eliminated by the use of a filter that blocks light of less than 370 nm.

As shown in Figure 7, the emission spectrum of the lamp / blue filter system used in the Dark Reader optical system contains less UV light than the standard fluorescent lighting used in most offices and laboratories. Because the Dark Reader transilluminator emits almost immeasurably low levels of light below 400 nm, there is essentially zero risk of UV radiation causing eye or skin damage, making it much safer to use than a traditional UV transilluminator.

3.1 In vitro DNA damage

It is well known that DNA samples undergo a number of reactions when exposed to UV light (see [28] for a review) including pyrimidine dimerization, breaks in the sugar-phosphate backbone and interstand cross-links and there have been several reports in the literature regarding the deleterious effects of UV irradiation on the biological integrity of DNA samples and cloning protocols. In an early study Brunk & Simpson [29] concluded that the extent of

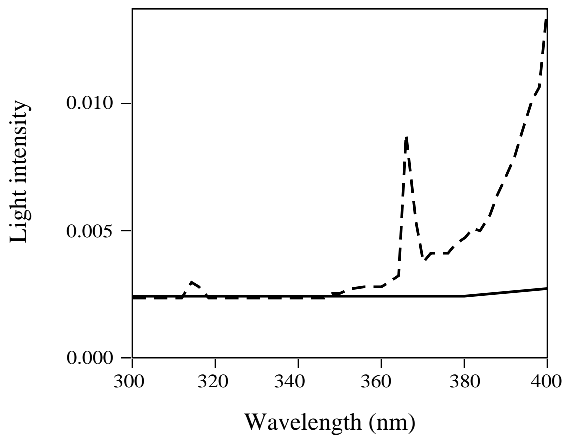

Figure 7. A comparison of the radiation intensity produced by standard overhead office lighting (dashed line) and a DR45 transilluminator (solid line) in the UV region.

DNA damage caused by 300 nm light was minimal. The extent of nicking was measured by velocity sedimentation of UV-exposed DNA samples in an alkaline sucrose density gradient and photodimer formation was measured by direct means. In both cases, the DNA samples were irradiated with UV light in solution, not in gels.

More recent studies make clear that there is, in fact, considerable damage to DNA samples that significantly impacts down-stream protocols. Cariello et al. [30] used denaturing gradient gel electrophoresis, which can resolve base pair substitutions, single base pair mismatches and methylation states, to reveal damage to a small (169 bp) dsDNA fragment within 10 sec of exposure to 300 nm light. Hartman [31] studied the effect of UV irradiation on several plasmids by measuring the transformation efficiency of treated samples. The results revealed up to a 100-fold reduction in transformation efficiency after exposure on a 302 nm UV transilluminator for less than a minute. Furthermore, the inactivation rate increased as a function of plasmid size. Hoffman [32] measured the extent of DNA damage caused by UV light using T4 endonuclease V (Endo V). This enzyme breaks the phosphodiester bond adjacent to pyrimidine dimers that are formed as a result of UV irradiation. A 1.2 kb fragment encoding the chloramphenicol acetyltransferase (CAT) gene was purified by gel electrophoresis and excised from the ethidium-stained gel using a 305 nm UV transilluminator. The excision took 10 - 20 seconds. The DNA was then incubated with Endo V and subsequently electrophoresed in a denaturing agarose gel. The results showed extensive formation of pyrimidine dimers in the samples that were briefly UV-irradiated. Furthermore, the number of chloramphenicol resistant colonies obtained upon transformation was reduced 50-fold showing that bio-activity can be severely compromised by exposure to UV light. In another recent study, Grundemann and Schomig [33] subjected plasmid DNA or cDNA samples to agarose gel electrophoresis and then exposed the gels for 20-45 sec to 312 nm UV light on the surface of a transilluminator while the fluorescent DNA bands were visualized and excised from the gel. The isolated DNA was used as a substrate for either transcription, transformation or PCR reactions. The efficiency of these procedures was reduced 2 - 3 orders of magnitude compared with unexposed DNA samples.

Because the Dark Reader transilluminator does not emit any UV light, it can be predicted that the extent of damage to DNA when viewed on a Dark Reader device will be drastically reduced compared to the damage produced by the use of a UV table. This is borne out by the results of the simple experiment shown in Fig. 8 in which supercoiled plasmid was exposed to DR or UV light for various times and then incubated with T4 endonuclease V. This enzyme excises any pyrimidine dimers that are formed in the DNA [32], generating the relaxed form of the plasmid which can then be resolved from the intact supercoiled form by agarose gel electrophoresis. It was found that as little as a 5 sec exposure to UV light is sufficient to allow conversion of almost 100% of the supercoiled plasmid (sc) into the relaxed form (rx) by endonuclease V. After 300 sec of UV exposure the DNA was completely fragmented. In contrast, a 300 sec exposure on the Dark Reader transilluminator resulted in no detectable DNA damage. This result suggests

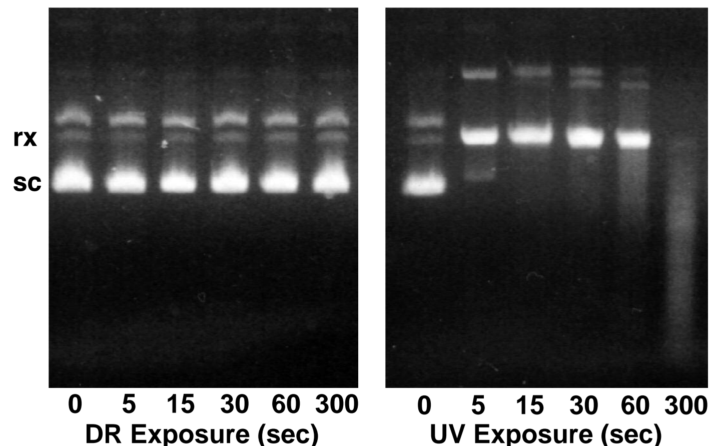

Figure 8. A comparison of the effect of exposure to DR or UV light. 100 ngof supercoiled (sc) plasmid was placed on either a Dark Reader transilluminator (DR) or a 312 nm UV transilluminator (UV) for various times. The DNA was then digested with T4 endonuclease V, which excises T:T dimers, and run on an agarose gel, stained with SYBR Green I stain and viewed. Upon exposure to UV light for 5 s, the supercoiled plasmid (sc) can be almost entirely converted to the relaxed form (rx) by endonuclease, indicating that pyrimidine dimer formation occurs extremely rapidly. After 300 s of UV exposure, the DNA can be almost completely fragmented by the endonuclease. In contrast, the DR-exposed plasmid remained intact over the entire time-course.

Table 3. Sensitivity of detection of dsDNA stained with various dyes after gel electrophoresis using either a Dark Reader or UV transilluminator and using various methods of detection

Dye	amount of DNA detected (pg)					
	CCD		Polaroid		Eye	
	DR	UV	DR	UV	DR	UV
SYBR Green	9	15	19	44	60	119
SYBR Gold	9	15	15	34	35	73
GelStar	9	15	15	31	44	120
ethidium bromide	623	89	500	125	2560	500

A summary of the minimum amounts of DNA visible in the CCD and Polaroid images shown in Figures 10, 11, 12 and 13. Also included are the amounts directly visible to the naked eye. For this purpose, gels were examined in a darkened room. Other experimental conditions are described in the legends to the relevant Figures. The sensitivity of detection was defined as the smallest amount of nucleic acid fluorescence that was clearly distinguishable in the image above background.

that the efficiency of downstream cloning protocols can be enormously improved by using a DR transilluminator, rather than a UV device, to visualize and excise DNA bands from gels after electrophoresis. This is confirmed by detailed studies on cloning efficiencies and DNA sequencing gel quality that will be the subject of a separate report (R. Mies, H. Daum, M. Fiandt, J. Jendrisak, L. Hoffman, manuscript in preparation).

Other area of research in which minimizing damage to DNA samples during processing is of prime concern and, therefore, the use of Dark Reader devices can be highly beneficial, include human population genetics [34] and mechanistic studies of DNA repair [35].

4 Some applications of Dark Reader technology

This section briefly describes some of the applications of Dark Reader devices with the new generation of fluorescent stains that are becoming popular for the ultra-sensitive detection of nucleic acids and proteins following (and even during) electrophoresis.

4.1 Nucleic acid stains

Ethidium bromide (EtBr) has long been the DNA stain of choice for many Molecular Biologists. However, it is gradually being replaced by a new generation of stains which are more sensitive and reportedly less toxic. These include Vistra® Green, GelStar®, PicoGreen®, OliGreen™, SYBR® Green I, SYBR Green II, and SYBR Gold stains.

The fluorescence enhancement of EtBr upon binding to nucleic acids is only on the order of 30-fold. Consequently, the background fluorescence from unbound ethidium dispersed throughout the gel is significant. The new generation of stains, on the other hand, are almost completely non-fluorescent in the absence of nucleic acids but, upon binding to nucleic acids, the fluorescence intensities are enhanced approx. 1000-fold, resulting in very high signal-to-background ratios [4]. Furthermore the quantum yields of the stain-nucleic acid complexes are 0.7 or greater, compared with 0.3 or less for EtBr-nucleic acid complexes [4].

4.1.1 SYBR® Green I stain

SYBR Green I stain was the first of the new generation of DNA stains introduced by Molecular Probes in 1994 [2, 3] and since then has been adopted by many researchers for the detection of DNA in electrophoretic gels. It has also found numerous other applications including the quantitation of PCR amplification of DNA [36] and DNA quantitation [37].

The fluorescence intensity of SYBR Green is enhanced over 2 orders of magnitude on binding to dsDNA. When used to stain DNA fragments separated by electrophoresis, the result is bright fluorescent DNA bands against a very dark gel background. Using a Dark Reader transilluminator it is possible to detect less than 100 pg of SYBR Green-stained DNA by eye (Table 3) and tens of picograms using a CCD or Polaroid camera system as shown in Figure 9.

Apart from its superior sensitivity, SYBR Green stain has a number of other advantages over EtBr:

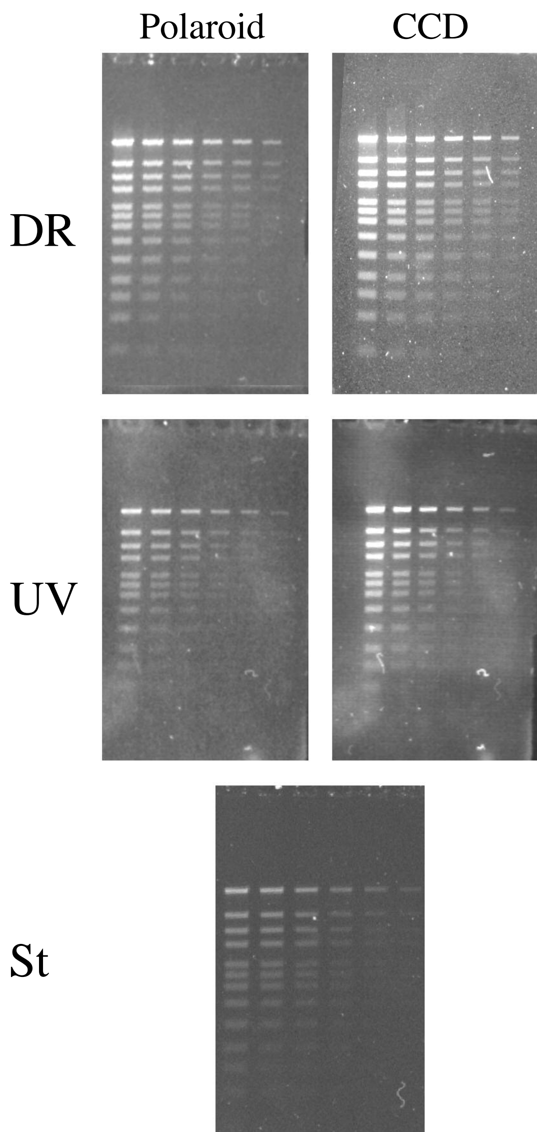

Figure 9. The sensitivity of DNA detection using SYBR Green I stain. A 2-fold dilution series of l DNA cut with StyI/SauI (Roche molecular weight marker IV) was subjected to electrophoresis in 1% SeaKem LE agarose (ADB, Inc.) in 1 x TAE buffer for 50 minutes at 5 V/cm. The total amount of DNA loaded per lane ranged from 12.5 ngdown to 0.39 ng. The several gels were subsequently stained with a 1:10,000 dilution of SYBR Green I stain in 1 x TAE for 30 minutes. A gel was then placed on either a 312 nm UV transilluminator or a Dark Reader transilluminator (DR180M, Clare Chemical Research, Inc.) (DR) and an image of the fluorescent DNA bands recorded using a Polaroid DS34 camera and 667 film (Pol) or an Olympus 3000 digital camera (CCD). In addition, a gel was imaged using a Storm 840 imager (St). When using the UV transilluminator, the cameras were equipped with a Wratten #15 plus #12 filter or a Wratten #9 (Kodak, Inc.) for photography. In addition, an IR-blockingfilter (#IF800, Clare Chemical Research, Inc.) was required for imaging with the CCD camera. The Dark Reader-illuminated gel was photographed with the amber screen provided. An f-stop of 2.8 - 5.6 was used for photography. The exposure time was varied as needed to achieve the longest exposure time that did not increase the background fluorescence level from the gel to such an extent that it masked the fluorescence from the DNA bands. Typical exposure times were 2 -5 s using the UV transilluminator and 4 - 6 s using the DR transilluminator.

It is much less mutagenic, as shown by researchers at Molecular Probes, Inc. who compared the mutagenicity of SYBR Green I stain with that of EtBr in Salmonella / mammalian microsome reverse mutation assays (Ames tests). They concluded that SYBR Green I stain is only a weak mutagen and appears to be much less mutagenic than EtBr [38]. One possible explanation for the reduced mutagenicity is that the SYBR Green stain does not intercalate between the DNA bases but, instead, binding involves surface or groove interactions.

A unique advantage of SYBR Green I stain is that, because it binds very tightly to dsDNA, it can be added directly to the DNA sample prior to electrophoresis and will remain bound during the separation run [39, 40]. This technique allows DNA fragments to be directly visualized as they migrate through the gel (Figure 10). Consequently, an electrophoresis run to be halted as soon as the desired DNA bands are separated - often within 30 minutes or less and electrophoresis results are thus obtained very quickly. Furthermore, unlike EtBr, which when used as a pre-stain must be added to the gel and the running buffer, SYBR Green stain need only be added to the DNA samples themselves. This drastically reduces the amount of dye required and virtually eliminates the risk of toxic spills. There is some retardation of the DNA (as there is with EtBr) during migration as shown in Figure 11. However, for loads below about 100 ng per band this effect is fairly

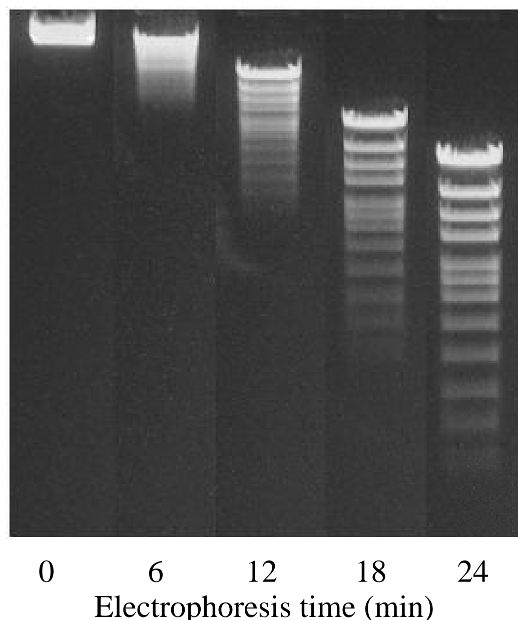

Figure 10. The utility of the Dark Reader ETU to monitor DNA fragment migration in real-time. 100 ngof lambda DNA cut with SauI/StyI was incubated briefly with a 1:1,000 fold dilution of SYBR Green I stain and loaded on a 1% agarose gel in a DR electrophoresis-transilluminator unit (ETU) (Clare Chemical Research, Inc.) and electrophoresed at 5 V/cm. The extent of migration of the DNA fragments was recorded using a CCD camera at the time points indicated.

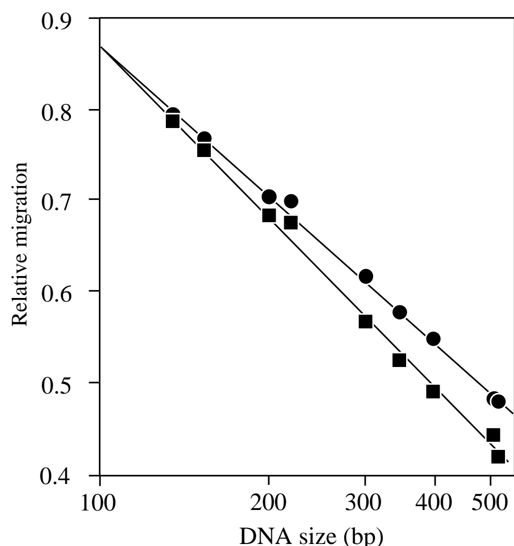

Figure 11. The effects of pre-staining samples with SYBR Green stain on the migration rates of DNA fragments. DNA molecular weight standards from Gibco Life Sciences (100 ng) were incubated with a 1:1,000 dilution of SYBR Green stain and run on a 12% non-denaturing polyacrylamide gel. A second aliquot of DNA, that was not incubated with SYBR Green stain, was run simultaneously. After electrophoresis, the gel was stained with SYBR Green (1:10,000) and the migration distance of the pre-stained (■) and post-stained (●) fragments measured.

linear and deviations are only seen at higher loading levels [40]. It should be noted that the sensitivity of DNA detection using a DR transilluminator or ETU is not quite as high when the samples are pre-stained (about 300 pg of dsDNA directly by eye using a DR device). Also, it has become apparent recently that the DNA products generated using some typical laboratory protocols, such as restriction digestion and PCR, may migrate anomalously if pre-stained with SYBR Green. The reasons for this are unclear at the present time. If accurate DNA fragment size determinations are required, the use of GelStar as a pre-stain (see section 4.1.3) is the preferred technique.

4.1.2 SYBR® Gold stain

SYBR Gold stain is one of the most sensitive of the new generation of dyes for the direct visual detection of dsDNA in gels [4] and it is possible to see less than 50 pg of dsDNA by eye using a Dark Reader transilluminator (Table 3). In combination with a CCD or Polaroid camera it is possible to detect as little as 10 pg of dsDNA as shown in Figure 12.

SYBR Gold stain is also reported to work well with RNA and ssDNA [4] and detection levels of 480 pg and 110 pg respectively have been reported. The stain enters gels very rapidly and major DNA bands can be seen within 5 minutes.

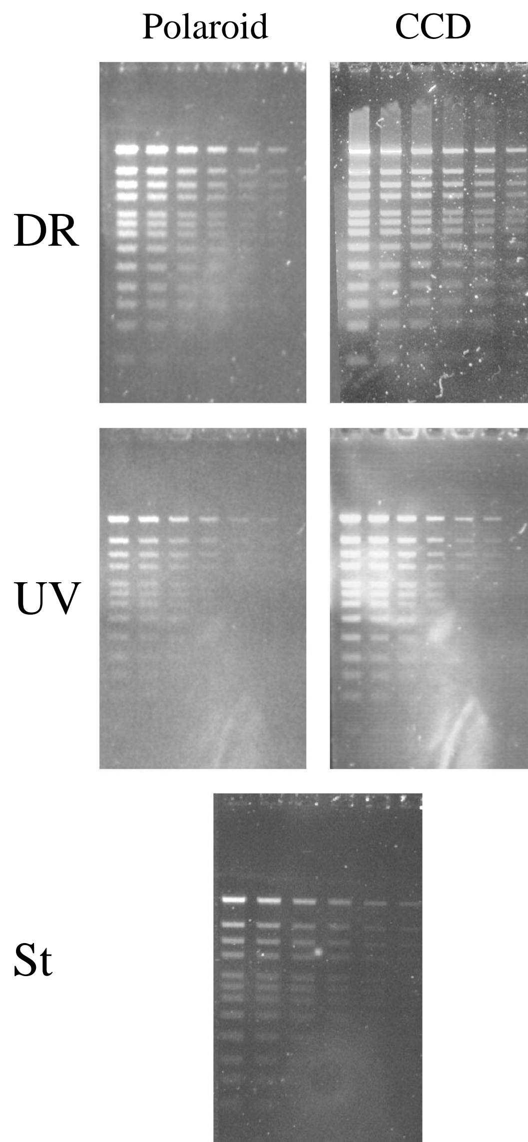

Figure 12. The sensitivity of DNA detection using SYBR Gold stain. The experimental conditions were identical to those described in the legend to Figure 9 except that the gels were stained in a 1:10,000 dilution of SYBR Gold stain.

Unfortunately, SYBR Gold stain cannot be used as a pre-stain as it severely retards DNA migration.

4.1.3 GelStar® stain

GelStar stain (BMA, Inc.) can be used for the sensitive detection of dsDNA, ssDNA, oligonucleotides and RNA in gels [5]. The detection limit of dsDNA stained with GelStar and viewed using a Dark Reader is comparable to that of SYBR Green and SYBR Gold stains as shown in Figure 13. GelStar stain can be used as a pre-stain, if added to the agarose, allowing DNA migration to be directly monitored. The presence of GelStar stain in the agarose during electrophoresis does not appear to result in any anomalous DNA migration behavior - a phenomenon that can occur with SYBR Green-stained DNA samples. Consequently, pre-

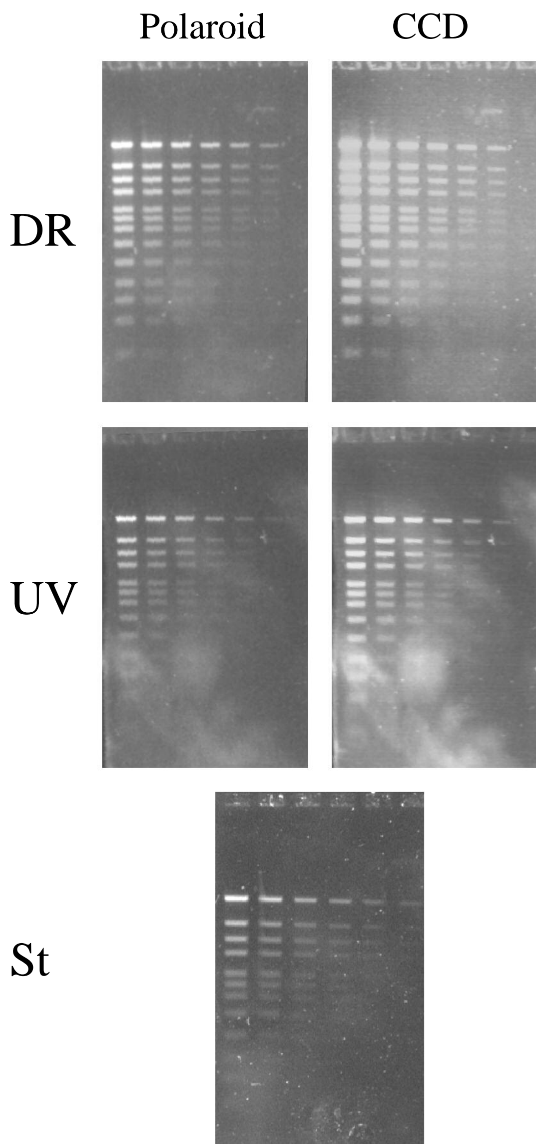

Figure 13. The sensitivity of DNA detection using GelStar stain. The experimental conditions were identical to those described in the legend to Figure 9 except that the gels were stained in a 1:10,000 dilution of GelStar stain.

staining with GelStar is the preferred technique for the quick and accurate determination of the sizes of DNA fragments.

4.1.4 Ethidium bromide

Ethidium bromide (EtBr) is intrinsically not as good a stain for the detection of DNA as the new generation of dyes described above. This is mainly due to the fact that the background fluorescence from unbound EtBr (i. e., fluorescence from EtBr free in the agarose gel) is relatively high. This is a consequence of the relatively small fluorescence enhancement of EtBr upon binding to dsDNA which is only round 20 - 30 fold. In addition, the quantum yield of EtBr is relatively low (~ 0.3) [4]. The background fluorescence problem is greatest when viewing EtBr-stained DNA gels with a DR transilluminator. As a result, Dark Reader transilluminators are not as sensitive as 300 nm UV-based devices for the detection of EtBr-stained DNA. (Figure 14 and Table 3.) The background problem can be minimized by using a lower concentration of EtBr to stain the gel. Staining a gel with an EtBr solution of 0.1 ug / mL (rather than the typical 0.5 - 1.0 µg / mL) significantly enhances the viewability of DNA bands. Though staining

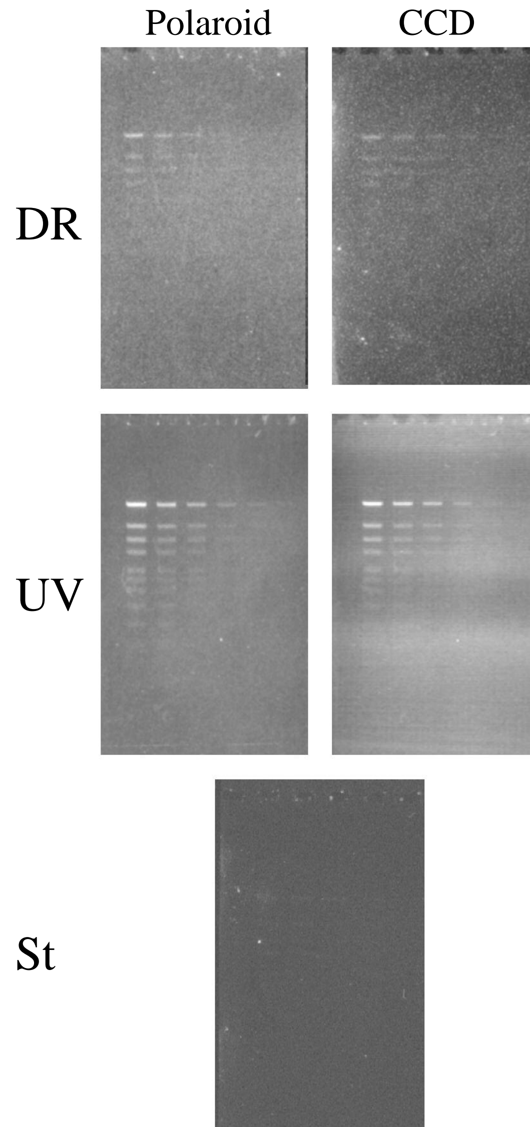

Figure 14. The sensitivity of DNA detection using EtBr. The experimental conditions were identical to those described in the legend to Figure 9 except that the gels were stained in a 0.1 ug / mL solution of EtBr. In addition, when using the UV transilluminator, the cameras were equipped with a Wratten #23A filter. In addition, an IR-blocking filter (#IF800, Clare Chemical Research, Inc.) was required for imaging with the CCD camera. The Dark Reader-illuminated gels were photographed with the amber screen provided together with an additional red-enhancing filter (#AF09, Clare Chemical Research). Typical exposure times were 2 - 6 s using the UV transilluminator and 5 - 10 s using the DR transilluminator.

times are a little longer (45 - 60 min), using these conditions, there is no need to destain the gel prior to viewing.

4.2 Protein stains

Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein mixtures the individual protein bands are typically visualized using either Coomassie brilliant blue R-250 [41], which is perhaps the most widely used protein stain, or silver staining [42] which provides a higher degree of sensitivity. Several new fluorescent protein stains have been recently developed by Molecular Probes, Inc. [6, 7, 8, 43]. These SYPRO® stains display excellent sensitivity similar to that of silver staining, less protein-to-protein variability than silver, a greater quantitation range, a simple one step staining procedure, and do not interfere with subsequent downstream characterization techniques. These stains are now becoming widely used in proteomics studies [44] and can be effectively detected using Dark Reader devices [43].

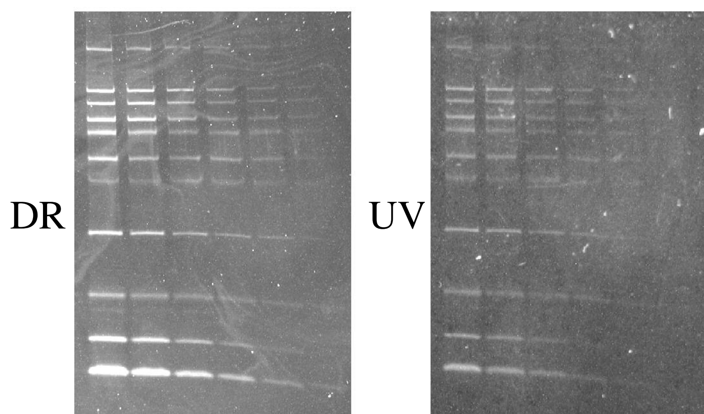

Figure 15. The sensitivity of protein detection using SYPRO Orange stain. A 2-fold dilution series of molecular weight standards (Molecular Probes, Inc.) was subjected to electrophoresis in a 0.05% SDS, 10-20% polyacrylamide gel in Tris-glycine buffer for 50 minutes at 100 V. The amount of protein per band ranged from 32 ng down to 1 ng. The gel was stained in 1:5,000 dilution of SYPRO Orange in 7.5% acetic acid for 30 minutes. The gel was then placed on either a 312 nm UV transilluminator or a Dark Reader transilluminator (DR180M, Clare Chemical Research) (DR) and an image of the fluorescent DNA bands recorded using an Olympus 3000 digital camera. When using the UV transilluminator, the camera was equipped with a Wratten #9 filter plus an IR-blocking filter (# IF800, Clare Chemical Research, Inc.). The Dark Reader-illuminated gel was photographed with the amber screen provided. An f-stop of 2.8 was used for photography. The exposure time was varied as needed to achieve the longest exposure time that did not increase the background fluorescence level from the gel to such an extent that it masked the fluorescence from the protein bands. Exposure times were 8 s using the UV transilluminator and 6 s using the DR transilluminator.

4.2.1 SYPRO® Orange stain

SYPRO Orange is a novel fluorescent stain for the detection of proteins separated by SDS polyacrylamide gel electrophoresis [6]. The staining procedure is simple, rapid and sensitive. The detection limit for Orange-stained proteins using a DR transilluminator is around 2 - 4 ng both by eye and using either a CCD or Polaroid camera (Figure 15). This level of sensitivity, especially by eye, is significantly greater than that obtained using a UV transilluminator (about 15 ng by eye).

The photo-bleaching of fluorophors upon exposure to light can become a significant problem, particularly when the experimental protocol is prolonged. This situation arises, for example, when proteins are being isolated from 2-D

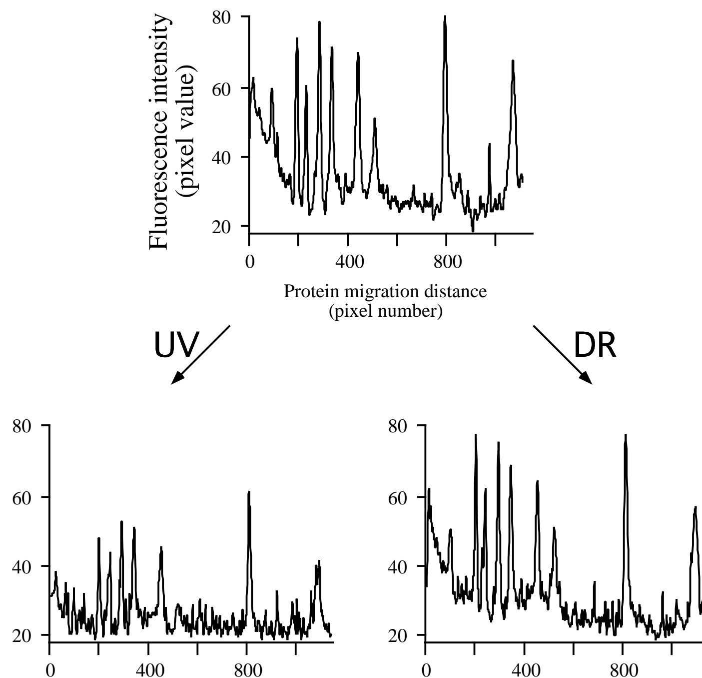

Figure 16. The extent of photo-bleaching of SYPRO Orange-stained proteins by UV and DR light was measured. An SDS polyacrylamide gel was loaded with 3 aliquots of protein molecular weight standards (15 ng per band), subjected to electrophoresis and then stained with SYPRO Orange (1:5,000). Two complete protein lanes were cut out from the gel and exposed on either a 312 nm UV transilluminator (UV) or a DR transilluminator (DR) for 8 minutes. The various protein lanes were then all photographed together on a DR transilluminator.

electrophoresis gels for downstream analysis. Clearly, if photobleaching can be minimized then the usable life of a gel can be extended accordingly, without the need to re-stain the gel. To determine the extent of photobleaching that occurs upon exposure of Orange-stained proteins to DR and UV light, samples were variously exposed for 8 minutes on either a DR or a 312 nm UV transilluminator. The results (Figure 16) show that UV exposure causes a ~40% decrease in the fluorescence intensity of the protein bands. Interestingly, some proteins appeared to be more significantly affected than others and were almost undetectable after 8 minutes of UV exposure. The DR exposure, on the other hand, resulted in a ~10% or less decrease in band intensity, indicating that the DR transilluminator is a more appropriate device for procedures that require extended exposure to exciting light.

4.2.2 SYPRO Ruby stain

The family of SYPRO Ruby stains are new, luminescent metal chelate protein stains that can be used to detect proteins in SDS-polyacrylamide gels, isoelectric focusing gels and on membranes [7, 43]. The dyes are maximally excited at 470 nm and the emission peak is about 610 nm. About 2 ng of SYPRO Ruby-stained protein can be detected directly by eye in an SDS-polyacrylamide gel using a Dark Reader transilluminator and about 8 ng after transfer to a PVDF membrane (unpublished results). This group of stains has become particularly popular for the detection of proteins following the 2-D electrophoretic separation of samples in proteomic studies.

5 Conclusions

The Dark Reader optical system provides for unique instrumentation to both view and image fluorophor patterns in electrophoresis gels and other biological samples with a sensitivity of detection that rivals that of any other optical system available. The key design features incorporated into the Dark Reader optical system include a relatively low power visible light excitation source and broad-band / long-pass filters. The inherent efficiency of this optical design enables users to directly view low level fluorescence emissions by eye in the most demanding applications including small Stokes' shift dyes and multiple fluorophors simultaneously.

The increasing awareness of the potential health hazards involved with the use of UV transilluminators is likely to result in their replacement, in the future, by DR devices. This is particularly true in university and high school teaching laboratories where student safety is of paramount importance. Even in research laboratories, the need for biological intact DNA samples to improve the efficiency of downstream cloning protocols, as well as the demand for the highest level of fluorophor detection will result in the increased use of DR transilluminators and other devices.

The unique filter construction used in the DR optical system and the ease with which they can be integrated into a wide variety of structures leads to almost complete freedom in instrument design. In combination with the low power requirements of DR lamps, many kinds of DR-based instrumentation are possible ranging from rugged, portable devices for field use, all the way up to entire room facilities. Indeed, several novel constructs are already under development by Clare Chemical Research, Inc.

This article is dedicated to the memory of S. W. Seville (1927-1997) who played an important role in the initial development of the Dark Reader.

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