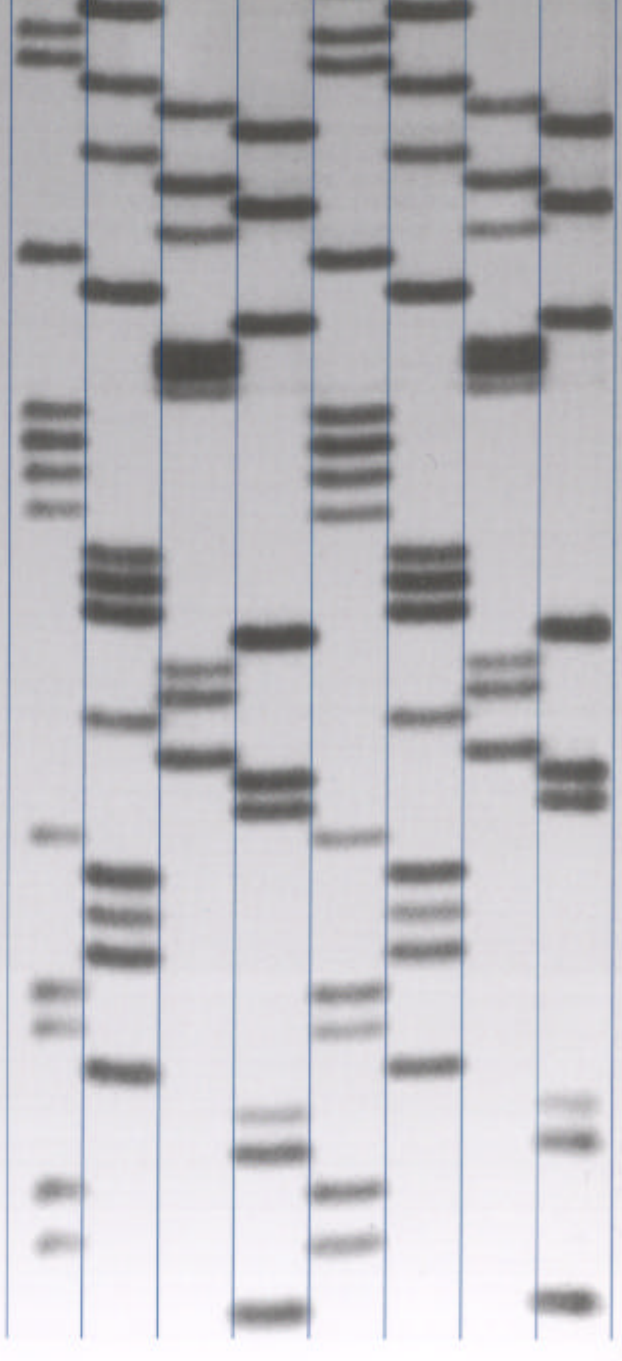
Review 23

Efficient Detection of Biomolecules by Autoradiography, Fluorography or Chemiluminescence

by R A Laskey



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Efficient Detection of Biomolecules by Autoradiography, Fluorography or Chemiluminescence

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Introduction

Techniques for biochemical fractionation have become more powerful and more accessible in recent years so their use is frequently limited by methods for detecting fractionated products. Labelling the sample with radioisotopes offers the twin advantages of specific labelling and extremely sensitive detection, but to exploit these advantages fully it is necessary to use the most efficient isotope detection methods.

For flat samples like slab gels or nitrocellulose sheets, autoradiography on radiographic film offers a unique combination of sensitivity and resolution. However, it suffers several limitations which restrict its sensitivity for most radioisotopes. Many of these constraints can be overcome by converting the emitted radiation to light, either by incorporating a scintillator into the sample (fluorography) or by enclosing film between the radioactive sample and a fluorescent intensifying screen. The choice of an optimal exposure procedure depends on the radioisotope, on the geometry of the sample and on the relative importance of sensitivity, resolution and quantitative accuracy for a particular exposure. Each of these priorities requires different exposure conditions. For example, the widespread practice of quantitating film images produced by intensifying screens introduces serious errors unless film has been hypersensitized by pre-exposure to a flash of light.

Recently non-radioactive alternatives have been developed for detecting specific proteins or nucleic acids after transfer to membranes by 'blotting'. These use enzymes to catalyse the chemiluminescent production of light. The light produced by luminol-based systems has a similar wavelength to that from intensifying screens or fluorographs and it can be recorded on radiographic film in short exposures at ambient temperature.

This review is intended to guide readers to the most appropriate exposure conditions for optimal sensitivity, resolution or quantitative accuracy when detecting radioisotopes by radiographic film or detecting non-radioactive molecules by enhanced chemiluminescence.

Summary of key concepts

Converting emitted radiation to light increases detection efficiency.

Impregnation of samples with a scintillator (fluorography) overcomes loss of signal due to self-absorption of weak β-emissions, for example ^{35}S, ^{14}C, ^{3}H or ^{33}P.

In polyacrylamide gels, scintillators (for example, Amplify™) can increase sensitivity 1000-fold for ^{3}H and over 10-fold for ^{35}S or ^{14}C.

Placing film between the sample and an intensifying screen increases detection efficiency of γ-rays and energetic β-particles, for example ^{32}P.

An intensifying screen can increase sensitivity 10.5-fold for ^{32}P or 16-fold for ^{125}I compared to direct autoradiography.

Enhanced chemiluminescence (ECL™) provides a safe, rapid alternative to radioactivity for detecting proteins or nucleic acids after transfer to membranes. ECL exposures are much shorter than most autoradiographic or fluorographic exposures.

Intensifying screens and fluorographs, but not ECL, require exposure at -70°C. All three require 'screen type' radiographic film sensitive to ultraviolet and blue light.

Pre-flashed film is essential for quantitating images from fluorography, intensifying screens or chemiluminescence and it improves sensitivity for weak signals.

Neither pre-exposure nor exposure at -70°C improves direct autoradiography without screens or scintillators.

Scintillators can be introduced into:
- acrylamide gels
- agarose gels
- nitrocellulose sheets
- paper chromatograms
- or thin-layer chromatograms

Several methods of impregnating acrylamide gels with scintillators offer similar sensitivity. They differ in resolution, convenience, safety and cost.

Drying of gels improves resolution for all isotopes.

Aqueous scintillants such as Amplify allow safe fluorographic impregnation in less than 30 minutes.

Table 1. **Radioisotopes used frequently in biochemistry**

Radioisotope	Emission		Half-life
	Type	Energy (max) MeV	
^{3}H	β	0.0186	12.43 years
^{14}C	β	0.156	5730 years
^{35}S	β	0.167	87.4 days
^{33}P	β	0.249	25.4 days
^{32}P	β	1.709	14.3 days
^{125}I	γ	0.035	60.0 days
	(+0.027 MeV X-rays + 0.030 MeV electrons)		
^{131}I	γ	0.364	8.04 days
	(+0.61 MeV β)		

Part I
Principles of detection using radiographic film

Radiographic film has been used for many years to detect radioactive molecules or more recently to detect specific non-radioactive molecules by probes which catalyse chemiluminescence. Although many radioisotopes will produce a direct autoradiographic image on film, sensitivity can be greatly increased by converting the emitted energy to light, superimposing a darker photographic image over the weak autoradiographic image. This is normally achieved by use of intensifying screens for high energy emissions or by use of organic scintillators for low energy emissions.

Although conversion to light can greatly increase sensitivity, the response of film to light is fundamentally different from its response to ionizing radiation. This is particularly a problem for recording low intensities of light, and it results in exposure of fluorographs or autoradiographs with intensifying screens at -70°C. It also results in a non-linear response of the film, seriously under-representing low levels and therefore restricting sensitivity and undermining quantitation [1,2].

The production of light by chemiluminescent detection systems has similar benefits of sensitivity, but similar constraints imposed by the non-linear response of film to light. Fortunately these constraints can be overcome easily for both chemiluminescence and radioisotope detection. The first part of this manual explains the principles and pitfalls and how to overcome them. First we consider the principles and practice of direct autoradiography in which the radioactive emissions blacken the film directly. Next we consider the advantages and disadvantages of converting ionizing radiation to light by intensifying screens or fluorographic scintillants. In addition we consider how strikingly similar principles and limitations apply to chemiluminescent systems.

Radioisotope detection by direct autoradiography

Principles of autoradiography

Radioactive isotopes were originally discovered by their ability to blacken photographic emulsions. Since that time, autoradiography on photographic film has remained a convenient method for radioisotope detection. Provided that the film makes close contact with a flat sample, autoradiography provides excellent spatial resolution and it avoids destruction of the sample. Silver halide crystals in the emulsion respond directly to (β-particles and γ-rays emitted from the sample. Each emission converts several silver ions to silver atoms to produce a stable latent image.

When the film is subsequently developed these few silver atoms catalyse the reduction of the entire silver halide crystal (grain) to metallic silver to produce an autoradiographic image of the radioisotope distribution. The energy from each β-particle or γ-ray is sufficient to render each grain it hits fully developable and many grains may be converted to the developable state by a single β-particle. Because only a single hit is required, the local blackening of the film for direct autoradiography is directly proportional to the amount of radiation reaching it. Thus in contrast to images obtained using fluorography, intensifying screens or chemiluminescence, the distribution of radioactivity in a direct autoradiograph can be quantitated accurately by scanning the film with a densitometer (as described in part II).

Single coated films with high silver content and fine grain size such as Hyperfilm^{TM}-(βmax (or Hyperfilm-^{3}H for tritium) are the most suitable for direct autoradiography.

Limitations of direct autoradiography

The usefulness of direct autoradiography is limited for most isotopes by inefficient transfer of their emission energy to the film. Direct autoradiography should be ideally suited to recording emissions from ^{14}C, ^{35}S or ^{33}P which emit (β-particles with maximum energies of 156, 167 and 249 keV respectively. Provided the β-particles are not absorbed within the sample, they are efficiently absorbed by the film. Most combinations of radioisotope and biochemical sample fail to match this ideal for two reasons. First, low energy emissions such as those from ^{3}H, ^{35}S and ^{14}C are internally absorbed within the sample, failing to reach the film. This problem is greatest for the very weak emissions of ^{3}H but it also decreases the autoradiographic efficiency for ^{35}S, ^{14}C or ^{33}P when these isotopes are embedded within the thickness of a sample such as a polyacrylamide gel. Second, the opposite problem limits sensitivity for highly energetic β-particles such as those from ^{32}P, or γ-rays such as those emitted from ^{125}I, ^{131}I, ^{51}Cr or ^{75}Se. These emissions pass through and beyond the film so that only a small proportion of their energy is captured and recorded by the film, most being wasted.

The problems of internal absorption within the sample and of inefficient absorption by the film can both be overcome by converting the emitted energy to light by the following procedures.

Applications suited to direct autoradiography

The following sections will show that conversion of ionizing radiation (electrons, β-particles, X- and γ-ray photons) to light can greatly increase sensitivity for most combinations of radioisotope and biochemical sample. What, then, is the value of direct autoradiography without scintillators or intensifying screens?

For penetrative emissions, such as those from ^{32}P or $^{125}1$, using an intensifying screen to convert the emissions to light decreases resolution. There is also a marginal loss of resolution when scintillators are used to detect ^{35}S- and ^{14}C-labelled samples. Therefore direct autoradiography remains the method of choice when optimal resolution is more important than optimal sensitivity, as is the case in DNA sequencing by the dideoxy method[3,4]. The extent of the advantage of improved resolution compared to the disadvantage of lower sensitivity will be illustrated below.

Conversion o f ionizing radiation to light for efficient detection by radiographic film

As explained above, the efficiency of direct autoradiography is limited by internal absorption of low energy emissions and by inefficient absorption of more energetic emissions by the film. Conversion of the emissions to light can solve both problems, greatly increasing the sensitivity of film detection.
The methods which are currently used to convert emissions to light all make use of organic or inorganic scintillators which emit photons of blue or ultraviolet light in response to excitation by β-particles or γ-rays. Thus they represent solid state equivalents of liquid scintillation counting. The light produced is recorded on radiographic film. Two classes of method exist which respectively overcome absorption of low energy emissions and increase the capture of highly penetrative emissions.

Absorption within a sample is overcome by impregnating the sample with a scintillator solution to achieve maximum contact between the isotope and the scintillator. In this way, even weak emissions can transfer their energy to scintillator molecules which then emit ultraviolet light. Provided the sample is translucent and colourless, the light can travel further than the original β-particle to form an image on the adjacent region of film (figure la). This procedure is called fluorography.

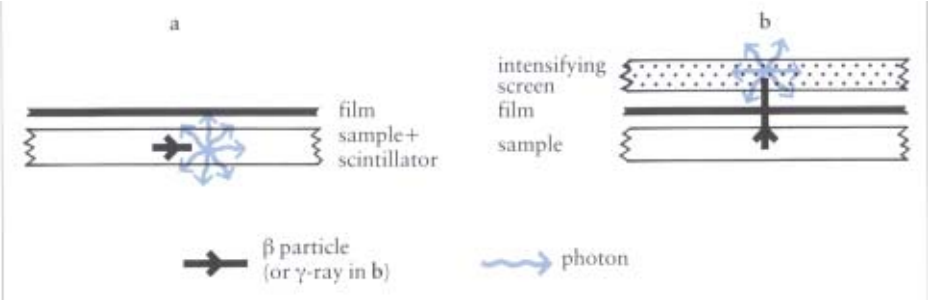

Figure 1. Conversion of (β-particles (or γ-rays) to light to overcome (a) absorption of weak emissions or (b) penetration of high energy emissions through and beyond the film.

Conversely, the wastage from penetration of γ rays or highly energetic β-particles through and beyond the film is overcome by placing a plastic screen incorporating a dense, inorganic scintillator, usually calcium tungstate, behind the film so that emissions which pass through are absorbed more efficiently by the scintillator with the production of light (figure 1b). The light produced superimposes a photographic image on the autoradiographic image. This procedure is a derivative of medical radiography and it exploits the intensifying screens which are designed and produced to minimize exposure of patients to X-rays. While intensifying screens may decrease the radiation hazard to patients, they expose their scientific users to the hazard of serious errors which can arise during the quantitative interpretation of images obtained using intensifying screens (see below).

Exactly the same problem arises when chemiluminescent detection systems such as ECL are used to detect specific molecules in gels or blots. Once again the light emitted produces a non-linear response of the film. Fortunately there is a simple way to overcome this problem.

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Advantage of converting radiation to light

There is a single advantage of converting β-particles and γ-rays to light for detection. This advantage is shown clearly in table 2. Conversion to light greatly increases the sensitivity of detection. For samples in polyacrylamide gels the increase in sensitivity varies from approximately 10-fold in the case of ^{32}P to over 1000-fold in the case of ^{3}H. The gain in sensitivity is not affected by the sample thickness for penetrative emissions such as ^{32}P or ^{125}l, but the gain observed for ^{14}C and ^{35}S in polyacrylamide gels may not be matched when these isotopes are detected on the surface of thin-layer chromatograms, or other samples when there is little self-absorption (see part II).

Table 2. Sensitivities of detection methods for radioisotopes in acrylamide gels (1.5mm thick)

Radioisotope	Dpm/cm^2 required for detectable image in			Improvement
	24 h (A=0.02) Direct autoradiography	Fluorography	Intensifying screen	by conversion to light
^{3}H	>8x10^{6}	8000	-	*>1000*
^{14}C	*6000*	*400*	-	*15*
^{35}S	*6000*	*400*	-	*15*
^{32}P	*525*	-	*50*	*10.5*
$^{125}1$	*1600*	-	*100*	*16*

Data from references 1 and 2. Direct autoradiography was performed on untreated film at 22°C. Other exposures were at -70 °C on film hypersensitized by pre-exposure to reach an absorbance of 0.2. Pre-exposure and-70 °C exposure are unnecessary for direct autoradiography. For fluorography and intensifying screens, blackening of correctly pre-exposed film at -70 °C is proportional to radioactivity in the sample and to exposure time, allowing other values to be calculated from this table. The newly available isotope ^{33}P was not used in these studies. Its efficiency of detection by direct autoradiography or fluorography will be higher than that of ^{35}S or ^{14}C.

Disadvantages of converting radiation to light

The gain in sensitivity which is achieved by converting emissions to light is partly offset by two disadvantages. As shown in figures 1 and 2 the resolution is decreased, since both the primary emissions and the secondary scintillations disperse. The extent to which resolution is decreased depends on the path length of the primary emissions and thus on the emission energy of the isotope. Fluorography of ^{3}H, which emits β-particles with an extremely short path length, gives excellent resolution (figure 3). ^{3}H-labelled bands spaced at only 1 mm intervals can be clearly resolved from each other in a gel impregnated with a scintillator. In contrast, figure 2 shows that resolution is lost when intensifying screens are used to increase the sensitivity of detection of ^{32}P, which emits highly energetic (β-particles. Figure 2 allows accurate assessment of the improved sensitivity and decreased resolution resulting from detection of ^{32}P by intensifying screens compared to direct autoradiography. For most purposes, such as resolving different sizes of protein or nucleic acid by gel electrophoresis, the loss of resolution is trivial compared to the gain in sensitivity. However, for certain critical applications such as in DNA sequencing gels, where poor spatial resolution of labelled bands limits the usefulness of the method, then the loss of resolution caused by intensifying screens is troublesome (figure 2).

The second disadvantage of converting ionizing radiation to light is potentially far more serious because it can result in misinterpretation of data. Once radioactive emissions have been converted to light the response of film is no longer linear. Low intensities of light produce disproportionately faint images. If it could not be corrected, this problem would eliminate the advantage of converting emissions to light since the intensity of light produced from scintillators or intensifying screens in normal biochemical work is too low to contribute significantly to the image on untreated radiography film at ambient temperature. Fortunately, as explained in the following sections, this problem can be completely overcome by pre-exposing film to a hypersensitizing light flash and then cooling the film to -70 °C.

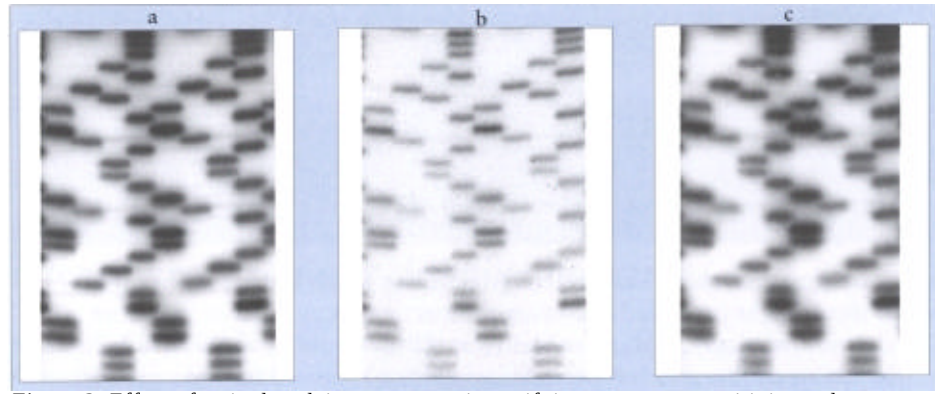

Figure 2. Effect of a single calcium tungstate intensifying screen on sensitivity and resolution of ^{32}P. Note that the screen increases sensitivity by approximately 10-fold (compare (b) with (c)) but decreases resolution (compare (c) with (a)). ((a) = overnight exposure without screens, (b) = 1 hour exposure without screens, (c) = 1 hour exposure at-70°C with screens, using preflashed Hyperfilm-MP).

Detection of light emitted by chemiluminescent probes

Various forms of blotting are increasingly used to detect specific proteins and nucleic acids after separation by electrophoresis. The separated molecules are transferred to a membrane by blotting and detected by a labelled probe. Although these methods were originally introduced for radioactive probes, non-radioactive alternatives such as enhanced chemiluminescence (ECL, Amersham) are being used to an increasing extent. Figure 4 illustrates the principles using the ECL Western blotting detection system [5,6]. In this case a secondary antibody is coupled to horseradish peroxidase which catalyses the oxidation of luminol in the presence of hydrogen peroxide. Luminol is converted to an excited state which emits light as it decays to the ground state. Light output is increased and prolonged by the use of enhancers, and it can be recorded on a blue-sensitive radiography film such as Hyperfilm-ECL.

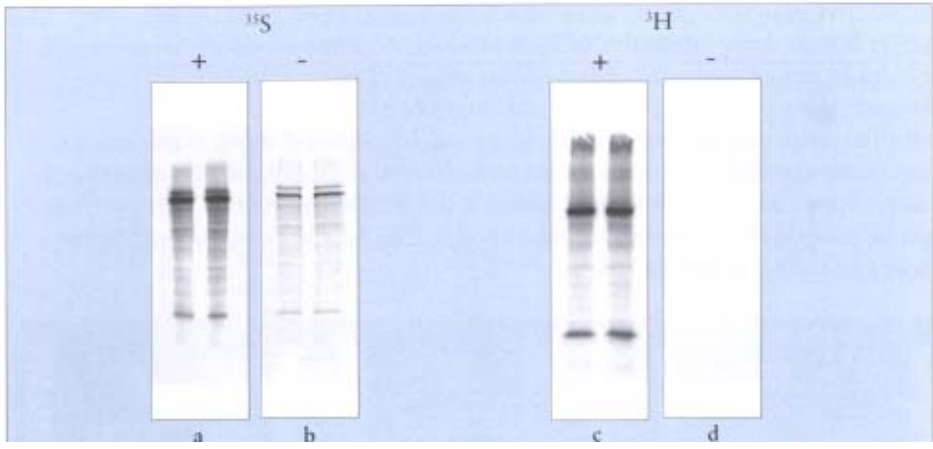

Figure 3. Effect of impregnating an acrylamide gel with Amplify on detection of proteins labelled with ^{35}S ((a) plus Amplify, (b) minus Amplify) or ^{3}H ((c) plus Amplify, (d) minus Amplify). Samples exposed to preflashed Hyperfilm-MP.

Exposure times for chemiluminescent systems are much shorter than those which record light produced following radioactive decay. Hence exposure at -70°C is not necessary. Indeed exposure at-70°C would be ineffective because it would inhibit the horseradish pcroxidase which is required for light production.

However the response of the film to light is still non-linear at low intensities. While this has the possible advantage that it gives a low background level, it results in under-representation of low intensities. Therefore, without an additional step, faint bands would not be detected and quantitation would be inaccurate. However, this problem is easily solved by pre-flashing film before use as explained overleaf.

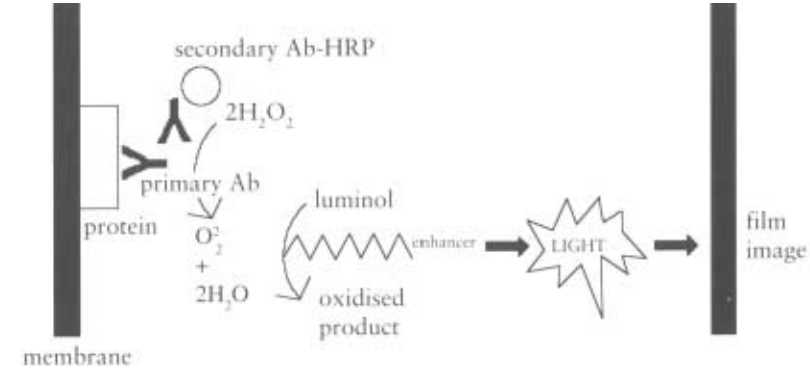

Figure 4. Principles of Western blotting with the ECL detection system. Note that ECL systems arc also available for nucleic acid probes.

Non-linear response of film to light from fluorographs, intensifying screens or chemiluminescence

Figure 5 illustrates the main difficulty which arises in detecting light emitted from fluorographs, intensifying screens or chemiluminescence. Photographic emulsions are disproportionately insensitive to very low intensities of light. Most exposures of biochemical samples involve low intensities compared to medical radiography. Therefore to obtain any image at all it is necessary to overcome the non-linear response of film to low intensities of light, a phenomenon known in the photographic literature as `low intensity reciprocity failure'.

The problem of under-representation of low intensities is greatest for very long exposures. Therefore it should apparently be much worse for the long exposures used for fluorographs or intensifying screens than for the shorter exposures used for enhanced chemiluminescence. In practice this difference is offset by exposure of fluorographs and intensifying screens at -70°C which greatly increases sensitivity of the film to very low intensities of light.

The under-representation of low light intensities can be largely overcome for all of these uses by pre-exposing film to an instantaneous flash of light, 'pre-flashing"[1,2]. As explained overleaf, this gives a linear film response to light, increasing sensitivity for low signals and allowing accurate quantitation of the film image.

In passing, it is interesting to note that similar procedures have been developed independently to overcome the same problem in another science, namely astronomy. Once again the problem is one of recording very low intensity light sources on photographic film, though in this case, film is hypersensitized by exposure to a mixture of hydrogen and nitrogen gas.

Effects of low temperature on sensitivity

Methods for impregnating chromatograms with scintillators have been developed since 1958[7] and a major advance in the techniques came when Luthi and Waser[8] reported that efficiency was greatly increased by lowering the temperature of exposure to -70°C. This improvement was attributed to increased light production from the scintillator at low temperature. However, the real reason why low temperature is so effective lies not in the behaviour of the scintillator, but in the response of the film[9]. As shown in figure 5 lowering the temperature to -70°C greatly increases the sensitivity of radiography film to low intensities of light[9].It has only marginal effects on light production by scintillators[10]. Thus it follows that the effects of temperature will be similar for light of a given wavelength whether it is produced from a tungstate screen or an organic scintillator. However, the temperature effect observed for light does not apply to the film's direct response to ionizing radiations and low temperature inhibits enhanced chemiluminescence.

Information on the optimal exposure temperature for detecting light by radiographic film is fragmentary, but the optimum appears to lie between 40°C and -90°C[1, 8-13]. The efficiency may possibly be slightly lower at -90°C than at-78.5°C, and it is much lower at-20°C than at-70°C. While it is not certain that -78°C is the actual optimum, it is clear that it is close to the optimal temperature for either intensifying screens or fluorography.

Effects of pre-exposing film ('pre-flashing') on sensitivity and quantitation

As shown in figure 5, lowering the temperature of the film to -70°C increases it's sensitivity to low intensities of light. However figure 6 shows that the relationship between blackening of the film and the amount of radioactivity in the sample is still not linear. This problem applies only when the emissions from the sample are in the form of light, such as those from intensifying screens[2], scintillators[1] or chemiluminescence. The reason why film responds so differently to light from its response to (β-particles will be explained in the following section. However the important practical consequence of that explanation is also shown in figure 6. Thus pre-exposure of the film to an instantaneous flash of light by-passes a reversible stage of latent image formation. This greatly increases sensitivity of the film to light emissions from small amounts of radioactivity and it can produce a linear relationship

between blackening of the film and amount of radioactivity (figure 6).

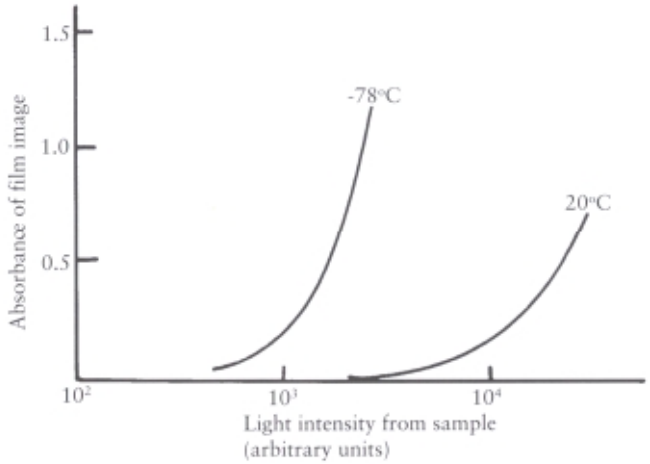

Figure 5. Effect of exposure temperature on sensitivity of radiographic film to light (data from reference 9).

The requirement for pre-exposure to obtain quantitative accuracy and optimal sensitivity applies equally to fluorography and intensifying screens. One report in the literature[13] states that pre-exposure has no effect on the efficiency of autoradiography using intensifying screens but this claim arose from studies using only large amounts of radioactivity in short exposures. Figure 6 shows that the effect of pre-exposure is observed clearly only when small amounts of radioactivity are studied and that it is easy to underestimate the effect of pre-exposure when it is assessed with large amounts of radioactivity.

If untreated film is used in long exposures, peaks will be artificially narrowed resulting in exaggerated resolution (figure 7) and small peaks will be seriously underestimated. For example, when comparing faint bands with dark bands in exposures of one week or more on untreated film, errors were found of 8-fold using ^{32}P and intensifying screens and >20-fold using ^{3}H and the scintillator PPO (references 1, 2 and Laskey and Mills unpublished). To hypersensitize film by pre-exposure, the flash of light must be short with a duration of about 1 msec. Longer flashes only increase the background without further hypersensitizing the film. A flash of this duration is provided by most photographic flash units as described in part II, or by Amersham's Sensitize™ unit in which the light source is already filtered to an appropriate intensity and wavelength.

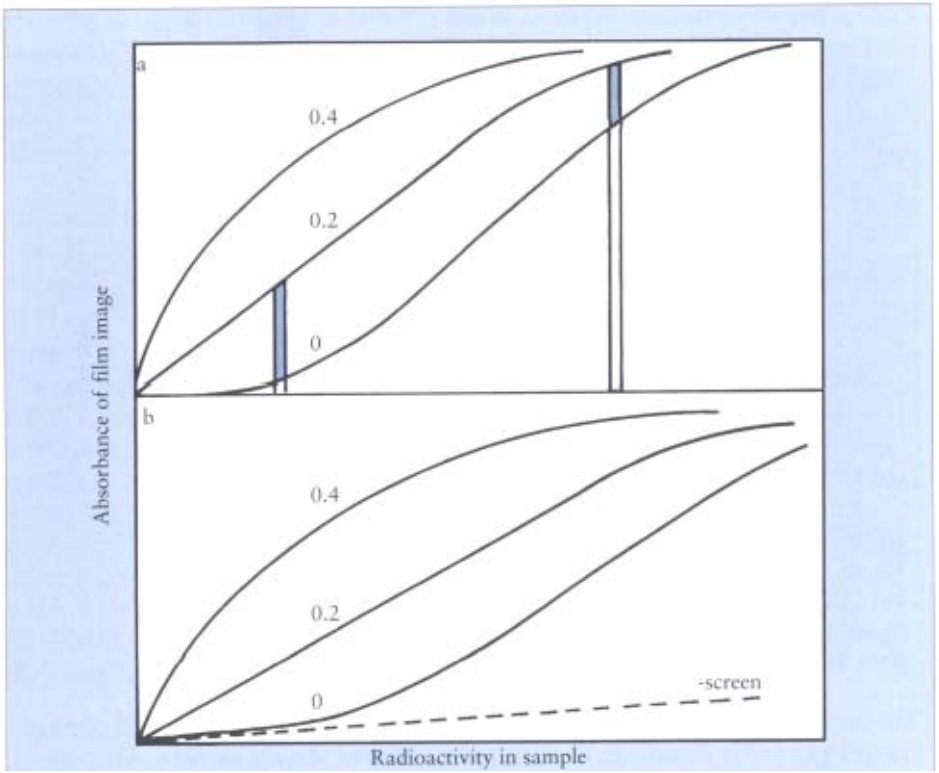

Figure 6. Requirement for pre-exposure to obtain a linear response of film to light from (a) a ^{3}H fluorograph or (b) ^{32}P with an intensifying screen. Pre-exposure absorbancies of 0, 0.2 and 0.4 above the absorbance of unflashed film are shown in blue. The vertical bars in panel a illustrate how the effect of pre-exposure (ratio of blue to white in vertical bars) is easily underestimated when it is assessed only with large amounts of radioactivity. The effect of pre-exposure on the film's response to light emitted by the ECL detection system is qualitatively similar.

A linear response of the film to light produced from the sample is obtained when the film has been pre-exposed sufficiently to increase its absorbance to 0.1-0.2 absorbance units above that of unexposed developed film. A further increase in the fog level reverses the deviation from linearity as shown in figures 6 and 7. Thus with higher levels of pre-exposure small amounts of radioactivity are over-represented. A further consequence is that peaks are broadened and resolution is decreased as shown in figure 7.

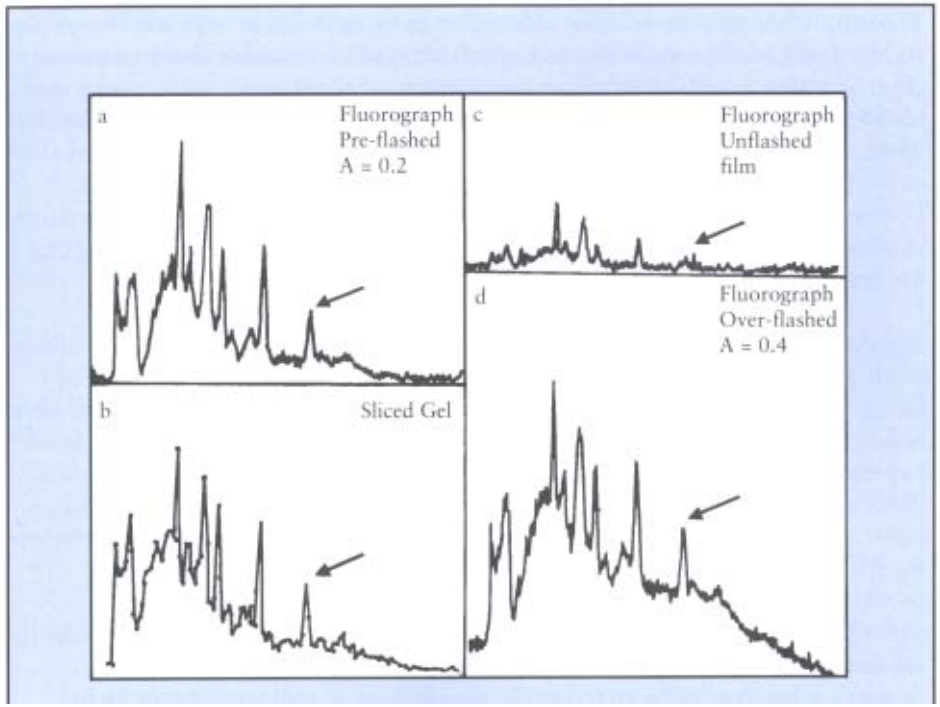

Figure 7. Effects of varied pre-exposure levels on images of ^{3}H distribution obtained by fluorography. Note that the arrowed peak is lost when unflashed film is used and exaggerated when overflashed film is used. (Data from reference 1).

Explanation of the effects on the film of low temperature and pre-flashing

Both pre-flashing and exposure at low temperature influence the non-linear response of the film to light. Neither has any effect on direct autoradiography in the absence of scintillators or intensifying screens[1,2]. Pre-flashing also corrects the non-linear response of film to the light produced by enhanced chetniluminescencc. The insensitive response to low intensities of light shown in figures 5-7 arises from the fact that the initial stage of latent image formation is reversible[14]. For a silver halide crystal (`grain') of the film to be developed by the developing solution it must accumulate several atoms of metallic silver (approximately 5 in average emulsions). These then catalyse the reduction of the entire silver halide grain (or large parts of it) to metallic silver by the developer. A single hit by a β-particle or γ-ray can produce hundreds of silver atoms, but a single hit by a photon of light produces only a single silver atom.

The crux of the problem is that although two or more silver atoms in a grain are stable, a single silver atom in a silver halide crystal is unstable and reverts to a silver ion with a half-life of about one second at room temperature. Since each photon produces only a single silver atom, the latent image can only accumulate when two photons arc captured by a grain within one second to produce a stable pair of silver atoms. At low light intensities this coincidence is rare. Lowering the temperature to -70°C increases the half-life of a single silver atom, increasing the time available for a second photon to arrive and thus increasing the sensitivity for low light intensities as shown in figure 5.

If instability is only a problem for single silver atoms, but not for pairs of silver atoms, then it should he possible to by-pass the reversible, unstable stage of latent image formation by pre-forming a stable pair of silver atoms in each silver halide crystal before the film is exposed to the sample. This is the principle of hypersensitizing film by pre-exposure to a flash of light. Once a stable pair of silver atoms is formed in each silver halide crystal each photon arriving has an equal chance of contributing to growth of the latent image (figure 8). Therefore the blackening of the film is directly proportional to the amount of light produced and hence to the amount of radioactivity in the sample, or of chemiluminescence generated. Obviously pre-exposure also increases sensitivity for small amounts of radioactivity or chemiluminescence but as shown in figure 6, it has negligible effects on large amounts of radioactivity, or large amounts of chemiluminescence.

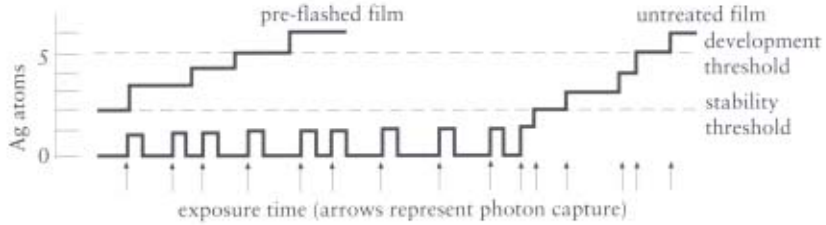

Figure 8. Effect of pre-exposure on the response to photon capture by individual silver halide grains in the film. A single silver atom is unstable. Pre-exposure to reach the stability threshold of 2Ag atoms per grain by-passes the reversible stage allowing immediate progress to the development threshold of approximately 5Ag atoms per grain. These catalyse development of the grain by the developer solution. Hence pre-exposure gives a linear film response to the intensity of light produced, allowing accurate quantitation.

It follows from figures 6-8 that pre-exposure should also by-pass the need for exposure at low temperature, since the temperature sensitive phase of latent image formation is by-passed. In practice this expectation is only partly fulfilled. utoradiographic exposure at -70°C is still approximately twice as sensitive as exposure at +20°C even when film has been pre-exposed.