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Production, fixation, and staining of cells on slides for maximum photometric sensitivity

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ABSTRACT

The need to detect increasingly low levels of antigens or polynucleotides in cells requires improvements in both the preparation and the staining of samples. The combination of centrifugal cytology with the use of glyoxal as cross-linking fixative produces monolayers of cells having minimum background fluorescence. Detection can be further improved by the use of a recently developed type of luminescent tags containing a lanthanide(III) ion as the light-emitting center. These novel tags are macrocyclic complexes functionalized with an isothiocyanate group to allow covalent coupling to a biosubstrate. The Eu(III) complex possesses a set of properties -- water solubility, inertness to metal release over a wide pH range, ligand-sensitized narrow-band luminescence, large Stoke's shift, and long excited-state lifetime -- that provide ease of staining as well as maximum signal with minimum interference from background autofluorescence. Luminescence efficiency studies indicate significant solvent effects.

1. INTRODUCTION

The detection and quantitation of molecules present at very low concentrations in cells require that the cells be attached to slides as a monolayer and stabilized with a fixative which maintains morphological integrity while producing minimal autofluorescence. Furthermore, the tags employed to visualize the monoclonal antibodies or nucleic acid probes must provide maximum signal with minimal background. The combination of centrifugal cytology with glyoxal as a fixative produces morphologically intact cells with minimal fixative induced autofluorescence. For these systems, the tag of choice is a luminescent europium(III) macrocyclic complex capable of producing emissions that are very narrow and have a long lifetime, and thus can easily be separated from any intrinsic fluorescence of the analyte.

2. CELL MONOLAYERS

2.1. Centrifugal cytology

Leif et al.[1] described centrifugal cytology, a method for centrifuging cells onto a transparent substrate, usually a conventional 1" x 3" glass microscope slide, and then wet fixing the cells while the centrifugal field holds and flattens them against the slide. Leif et al.[2] demonstrated that centrifugal cytology could be used successfully to prepare monolayers from cervical scrapes. A special centrifuge bucket was constructed to facilitate the wet fixation of cells (Figure 1). Subsequent publications established its utility for urine[3], ocular cells[4], breast aspirates[5], and other cells[6]. Cowden and Curtis[7], used the Leif Centrifugal Cytology Buckets[8] with rat thymic lymphocyte preparations. After Hoechst 33258 staining, these authors obtained a CV of 2.9% from a sample of 121 cells with a mean relative intensity of 100. With DAPI, they obtained a CV of 3.1% from a sample of 308 cells, with a mean relative intensity of 84.

2.2. Fixation with glyoxal

Leif et al.[9] developed a means to prepare stabilized leukocytes for immunofluorescence analysis. Part of the procedure involved the use of glyoxal as a fixative. Glyoxal, the simplest of the dialdehydes, is a fixative stronger than formaldehyde and weaker than glutaraldehyde; compared to the latter, it has the very significant advantage of not forming a fluorescent adduct with the substrate. Figure 2 shows human leukocytes prepared as described in Example 7 of

Leif et al.[9]. Briefly, the lymphocytes in human whole blood were labeled with a CD8 specific monoclonal antibody and DAPI, the erythrocytes selectively lysed with saponin, fixed with glyoxal, and the cells prepared on a slide with the Centrifugal Cytology Bucket..

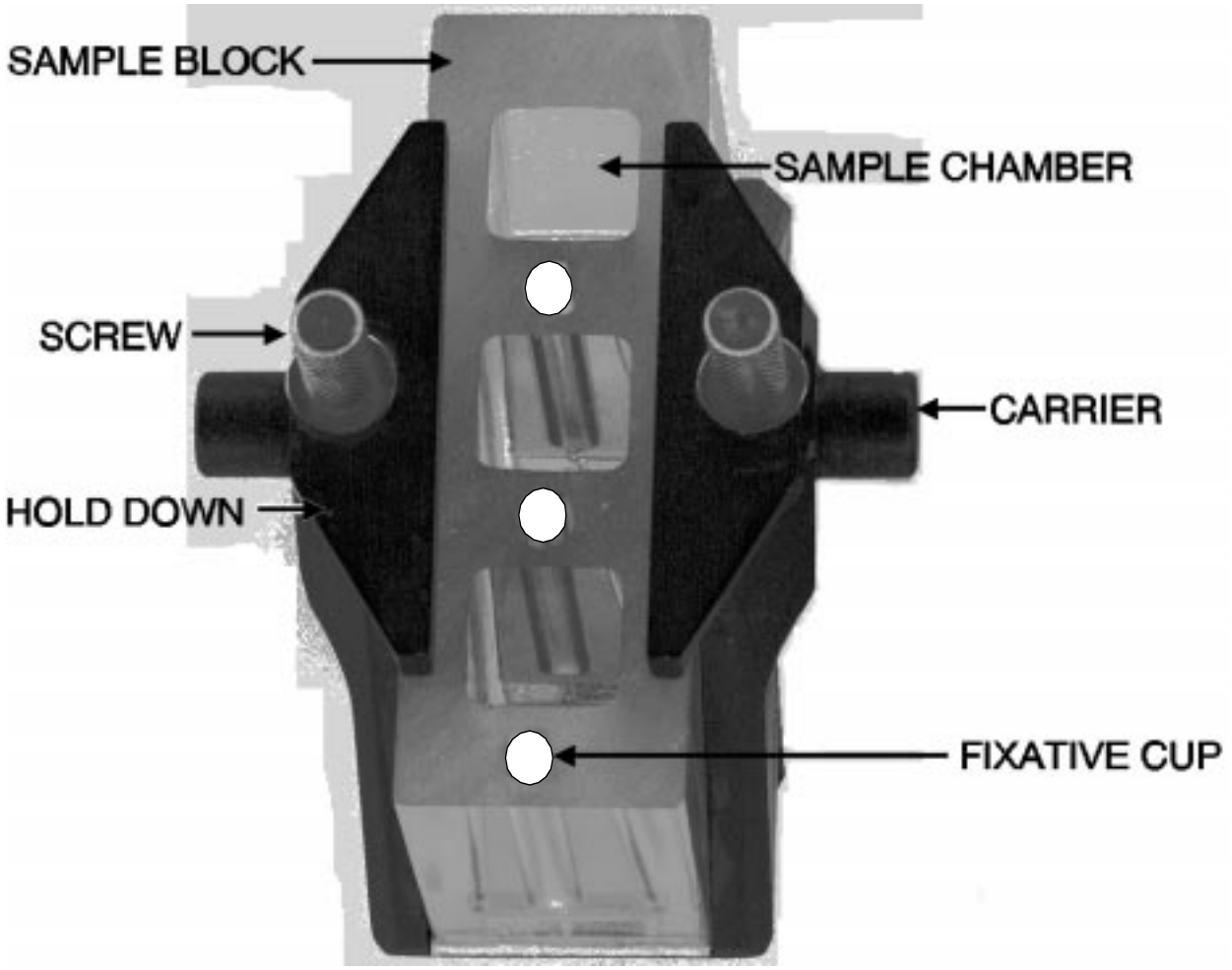

Figure 1, Leif Centrifugal Cytology bucket[8]. The bucket is assembled as follows: the hold downs are rotated 180^{o} from the position shown in the figure, a gasket is inserted into the base of the carrier, a microscope slide is placed on top of the gasket, the sample block is placed on top of the slide, the hold downs are rotated 180^{o} and the screws tightened. The cell containing suspension is put into the sample chamber. After the first centrifugation, the supernatant is removed and fixative is added to the fixative cups. While the buckets are centrifuged a second time, the fixative covers the cells. Thus fixation occurs under the action of the centrifugal force.

3. QUANTUM DYES AS LUMINESCENT TAGS

3.1. General properties of Eu(III) macrocyclic complexes

The synthesis and structural characterization of europium(III) complexes of six-nitrogen-donor macrocyclic ligands have been described[10, 11, 12]. Similar to many other Eu(III) derivatives, these Eu(III)-macrocycles exhibit narrow-band, long-lived, ligand-mediated luminescence upon irradiation with ultraviolet light of appropriate wavelength[12, 13]. Their chemical properties, however, are unusual. The Eu(III)-macrocycles are soluble in water as well as in organic solvents and -- unlike most other water-soluble lanthanide(III) complexes -- remain undissociated in very dilute solutions

(micromolar or less). Furthermore, the Eu(III)-macrocycle entities are inert to metal release or ligand hydrolysis over a wide pH range and in the presence of strongly competing ligands. In contrast, any exocyclic ligands (counterions or solvent molecules) are labile and readily exchangeable.

Figure 2. Centrifugal Cytology dispersion of Coulter Clone(R) T8-FITC (CD8) and DAPI stained, glyoxal fixed human leukocytes. The two cells above and to the right of the printing have an immunofluorescent rim. Because of the undetectable background cytoplasmic fluorescence, most of the other cells appear to be bare nuclei. The slight shift of the immunofluorescence halo is an artifact, presumably due to the use of two exposures, the first to obtain the weak fluorescein immunofluorescence and the second to obtain the very strong DAPI DNA fluorescence.

The original photograph was made with color slide film and the black and white print was made with high contrast paper in order to permit the immunofluorescence halos to be seen. The chromatin structure is lost because of the over exposure of the nuclei.

3.2. Attachment of functionalized Eu(III) macrocyles to biosubstrates

When the Eu(III)-macrocyclic complexes are functionalized[14, 15] with peripheral reactive groups (e.g. NH_2 or NCS), they may be conveniently coupled to any desired biosubstrate by standard procedures. The attachment of the Eu(III)-NCS-macrocycle to avidin has been reported[16] and the resulting conjugate exhibited both Eu(III) luminescence and biotin-binding ability[16]. It should be noted that the functionalized complexes are formed by the 2:2 cyclic Schiff-base condensation of 2,6-diacetylpyridine with carbon-substituted ethylenediamine and hence can exist as constitutional as well as and steric isomers (Figure 3). Several isomeric complexes were indeed obtained, but they were extremely similar in all their properties except nuclear magnetic resonance[17]; accordingly, no separation was required.

3.3. Eu(III) macrocycles as luminescent tags

It has been pointed out[15, 16] that the functionalized Eu(III)-macrocyclic complexes possess a combination of properties that makes them uniquely suitable as luminescent tags (“Quantum Dyes”). At present, the major uses proposed for the Quantum Dyes are as tags in immunoassays and in the image analytical cytology of fixed specimens. The conditions for these two types of measurements are very different. Immuno-assays, as well as the microscopy of living cells, are conventionally performed in aqueous solution. In contrast, the microscopic analysis of fixed cells at maximum optical resolution requires a mounting medium with a refractive index which is approximately equal to that of the slide and coverslip. Conventionally, glycerol solutions are employed for this purpose. The following section reports the results of a study directed at optimizing the luminescence intensity of the Eu(III)-macrocycles under the conditions required by each type of measurement.

3.4. Rationale for the optimization of the Eu(III)-macrocycle luminescence

Irradiation of Eu(III) complexes with ultraviolet light results in intense luminescence when two chief conditions are satisfied:

(1) The Eu(III) ion must be coordinatively bound to one or more ligands, usually π-bonded organic systems capable of acting as efficient photo-traps. These ligands absorb the ultraviolet radiation and, if their excited state is of appropriate level, transfer a portion of the absorbed energy to the Eu(III) center, exciting it to its emission level(s). In general, the most effective luminescence-inducing ligands for Eu(III) are chelating ß-diketonates with absorptions in the 340-380 nm range.

(2) The Eu(III) ion, once excited to its emission level(s), must be protected from deactivation (luminescence quenching) due to vibrational interaction by with the environment. Water is an especially effective quencher and for this reason aqueous solutions of Eu(III) complexes are only weakly luminescent unless protective additives are also present.

To establish the conditions for optimum luminescence of the Eu(III) macrocyclic complexes with respect to the above requirements, we first screened several ß-diketonates with absorption maxima in the desired 340-380 nm region, using as model the X-ray analyzed {Eu($C_{22}H_{26}N_6$} triacetate complex (abbreviated as Eu-MAC). We found the anion of 1,1,1-trifluoro-4-(2thiophene)-2,4-butanedione to be the most effective luminescence enhancer, followed closely by the anion of 1,1,1-trifluoro-4-(2naphthyl)-2,4-butanedione. The latter is the enhancer used by Hemmila et al.[18]. All measurements described below were accordingly performed using 1,1,1-trifluoro-4-(2thiophene)-2,4-butanedione as the enhancer precursor. A model of the Eu-MAC-enhancer species is shown in Figure 4.

Figure 3. Schematic formulas of the two constitutional isomers of symmetrically disubstituted six-nitrogen-donor macrocyclic complexes (M = Eu, X = -CH_2-C_6H_4-NCS). Since the carbon atoms to which the X substituents are attached are chiral, each constitutional isomer also exists as stereoisomers.

3.5. Selection of buffer and solvents

The next concern was to find a buffer that would provide the pH range (7.4-8.0) desired for bio-measurements without either causing precipitation of the Eu(III)-macrocycle or competing as a ligand with the ß-diketonate anion. Buffers containing trinegative ions (e.g. phosphate and borate buffers) proved unsuitable as they tended to cause precipitation; whereas, carbonate-bicarbonate and N-substituted aminoacid buffers did not cause this problem. Initial luminescence studies were performed in aqueous carbonate/bicarbonate buffer, since this is a common medium for bio-systems and it is also used in the coupling of the isothiocyanate functionalized macrocycles to the lysine amino groups of proteins. In this medium, however, the luminescence intensity for 2.50 x 10^{-6} M Eu-MAC (Eu($C_{22}H_{26}N_6$)($CH_3COO)_3(H_2O)_4$, F.W. = 752.1), in the presence of 1.13 x 10^{-5} enhancer (1,1,1-trifluoro-4-(2-thiophene)-2,4-butanedione, "Thenoyltrifluoroacetone", F.W. 222.2, Aldrich T2,700-6), was too low to be useful. Substitution of the carbonate/bicarbonate by

0.10 M tricine (N-tris[Hydroxymethyl]methyl-glycine, Sigma T-0377), accompanied by the addition of 5.0% v/v poly-ethyleneglycol (PEG, average M.W. 1,500, Aldrich 20,243-6), 0.1% Triton non-ionic detergent (Triton X-100, Aldrich 28,210-3), and 0.10% sodium azide resulted in a considerable increase in luminescence. Quantitative measurements at a higher concentration (1.25×10^{-4} M Eu-MAC, 2.50×10^{-4} M enhancer) showed the integrated emission intensities of the tricine-PEG-azide solution to be higher than those of the carbonate/bicarbonate solution by a factor of 48 (for the $^5D_0D^7F_1$ transition at 590-600 nm) and of 115 (for the $^5D_0D^7F_2$ transition at 610-625 nm). Slight changes in pH of the bicarbonate buffer also have a significant effect on light emission. Tricine was therefore the buffer of choice for the comparative study of the Eu-MAC-ß-diketonate luminescence in different solvents. The Triton X 100 will have to be replaced if viable cells are to be measured, but can be used for other procedures such as immuno-assay. Each solution contains 2.50×10^{-6} M $Eu(C_{22}H_{26}N_6)(CH_3COO)_3(H_2O)_4$ and 3.13×10^{-5} M enhancer (1,1,1-trifluoro-4-(2-thiophene)-2,4-butanedione). The composition of the "Hemmila standard" is given in the text.

Figure 4. Computer generated model of the Eu-MAC-enhancer complex, showing two chelating ß-diketonates bound to Eu(III) on opposite sides of the macrocycle. The model was obtained with Evans-Sutherland's SYBYL 5.41 program, utilizing the atom coordinates from X-ray data for the Eu-MAC moiety[12]. The bond lengths and angles for 1,1,1-trifluoro-4-(2-thiophene)-2,4-butanedionate and the Eu-O(diketonate) distances were set at the values reported in the literature for other lanthanide-ß-diketonate complexes[19]. The model shows that there are no forbidding van der Waals repulsions between the two axial diketonates and the macrocyclic ligand.

The following solvent systems were investigated (Table 1), each containing 2.5×10^{-6} M Eu-MAC, 1.13×10^{-5} M enhancer, 0.10 M tricine, and 0.10% sodium azide (1.0 mg./mL.): (1) Water 95%, polyethyleneglycol (M.W. 1,500) 5.0% v/v, Triton X-100 0.10%; (2) water 20%, glycerol 80%; (3) Water 20%, ethyleneglycol 80%; (4) Water 10%, ethanol 90%.

In order to obtain a consistent set of data, all luminescence measurements of the Eu-macrocycles were compared, at the same Eu(III) concentration and using identical instrumental settings, to the values obtained for the solution

described by Hemmila et al.[18], which is used in the DELFIA method of analysis[20]. Soini and Hemmila[21] and later Soini and Lovgren[20] have described an analytical procedure involving the Eu(III) complex of the polyamino-carboxylate ligand DTPA[22], which is non-luminescent in aqueous solution. In this procedure, the Eu-DTPA chelate was first decomposed with acid and the solubilized Eu(III) was then complexed with a ß-diketonate in a micellar phase as described by Hemmila et al.[18]. This homogeneous aqueous phase technique does not permit localization of the chromophore and thus is unsuited for microscopy. However, because it is a standard procedure which does produce very bright luminescence, it was chosen as the reference for our studies. The aqueous Hemmila solution (pH 3.2) used as comparison standard contained: 2.5 microMolar $EuCl_3$-$6H_2O$ (Aldrich 21,288-1), 0.1 M NaAcetate, 0.1 M potassium hydrogen phthalate (Fisher P243), 15 microMolar 4,4,4-Trifluoro-1-(2-napthyl)-1,3-butanedione (Aldrich 34,363-3), 50 microMolar Trioctylphosphine oxide (Aldrich 22,330-1), 0.1% Triton non-ionic detergent (Triton X-100, Aldrich 28,210-3).

Table 1 summarizes the observed emission intensities of the solutions examined in the present study, expressed as percentages of the intensity of the "Hemmila standard". It should be noted that emission intensities were measured for both the $^5D_0$ D $^7F_1$ (590-600 nm) and the $^5D_0$ D $^7F_2$ (610-625 nm) Eu(III) transitions. Although the former emission is by far the less intense, it does in each case represent a greater percentage of the "Hemmila standard", most likely as a result of the different symmetries of the E(III) environment in the two species.

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Table 1: Relative Emission Intensities of Europium(III)- Macrocycle-Enhancer Model System in Various Solvents.[1]

Solvent	% of Hemmila 610-625 nm	% of Hemmila 590-600 nm
5% Polyethyleneglycol (MW 1,500), 95% water	1.8	14.7
80% Glycerol, 20% water	3.2	16.4
80% Ethyleneglycol, 20% water	8.6	45.3
90% Ethanol, 10% water	9.6	53.3

[1] Each solution contains 2.50 x 10^{-6} M $Eu(C_{22}H_{26}N_6)(CH_3COO)_3(H_2O)_4$ and 3.13 x 10^{-5} M enhancer (1,1,1-trifluoro-4-(2-thiophene)-2,4-butanedione). The composition of the "Hemmila standard" is given in the text.

The results with the $^5D_0$ D $^7F_2$ (610-625 nm) Eu(III) transition, which is the major signal source show that the luminescence of the Eu-MAC-enhancer system is highly dependent upon the choice of both buffer and solvent. The emission intensity increases dramatically in the absence of those buffers that contain anions, such as carbonate, capable of competing with the ß-diketonate enhancers as ligands for Eu(III). The emission intensity also increases greatly in the less hydroxylic solvents. However, vibrational deactivation by interaction with the -OH groups of solvent molecules can not be solely responsible for the energy loss, since substitution of D_2O for H_2O as the solvent had been reported to results only in a three-fold increase of the Eu-MAC excited-state lifetime[13].

4. Conclusions

Cell monolayers with good morphology and minimal fixative induced autofluorescence have been produced by employing centrifugal cytology with glyoxal fixation. The low quantum yield of the Eu-macrocycle in aqueous medium probably precludes its use as a tag for the observation and measurements of live cells. However, this complex can be

used in conventional fluorescence microscopy, providing ethyleneglycol replaces glycerol as the mounting medium. Other uses of the Eu-macrocycle as a tag, such as immunodiagnostics, are feasible providing the measurements are performed in a nonaqueous solvent such as ethanol.

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6. References

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