Chapter 31
Purification and coupling of fluorescent proteins for use in flow cytometry

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The most commonly employed fluorescent dyes in immunofluorescence are fluorescein (green) and rhodamine (red). Both have the advantage of ease in labelling, which involves simply mixing an activated dye reagent with the antibody of interest [1]. Filter sets have been developed that permit rapid sequential observation of a double labelled sample for fluorescein and then rhodamine fluorescence. However, for microscopy (which is typically carried out using a mercury arc lamp), fluorescein is not optimally excited. Rhodamine is well matched to the arc excitation and so is commonly employed where highest sensitivity is required.

In laser-based flow cytometry, the situation is to some extent reversed, i.e. fluorescein is well matched to the 488 nm argon-ion laser line, whereas rhodamine is suboptimally excited by the 514.5 nm argon-ion laser line. In addition, the wavelengths of fluorescein and rhodamine emissions are not well separated, which is not a problem for sequential measurements, but severely limits simultaneous measurements. These facts have restricted the use of dual staining to make correlated measurements in flow cytometry. With careful filtering and electronic compensation it is possible to derive independent signals from the fluorescein/rhodamine pair [2], but because of compromises in accommodating both dyes, signal quality for both stains is degraded compared to either one used individually in an optimized system.

More recently, the addition of a second laser on commercial flow cytometry systems has permitted the development of optimized dual-staining correlated analysis. Either a krypton laser is used to excite the x-rhodamine derivative [3] or an argon-ion/rhodamine 6G dye laser [4] is used to excite the sulphorhodamine dye, 'Texas red' [5]. These systems give independent signals, both optimized for the individual dye, and the latter system in particular permits very high quality dual staining measurements [6]. The obvious disadvantages of this approach are the requirements of a complex, expensive set-up in order to use the fluorescein/Texas red combination.

An alternative to a two-laser system for obtaining dual staining data in flow cytometry is the development of new dyes matched to available argon-ion laser lines. This has occurred in the past year with the application of fluorescent photosynthetic accessory pigment proteins to immunofluorescence methods [7]. These proteins, called phycobiliproteins [8,9], are found in red algae and cyanobacteria [10] (see Table 31.1) where they serve to transfer energy from blue light to the chlorophyll photosynthetic system (not excited well in the blue) [11]. Phycobiliproteins are well suited as fluorescent dyes since they possess many fluorescent groups (bilins) of several distinct types per protein. This results in a broad excitation range and a very large extinction coefficient compared to small organic fluorochromes (see Table 31.2).

Phycobiliproteins can be divided into three major groups: the phycoerythrins (PEs) with a molecular mass of 240 kDa, having major excitation peaks between 490 nm and 560 nm and emission at 570 nm; the phycocyanins (PCs) with a molecular mass of 240 kDa, having good excitation from 580 nm to 620 nm and emission at 640 nm; and the allophycocyanins (APCs) with a molecular mass of 110 kDa, having good excitation between 600 nm and 650 nm and emission at 680 nm. As can readily be seen from the excitation/emission spectra (Fig. 31.1), the orange fluorescence of PE can be excited with reasonable efficiency by the 488 nm line of the argon-ion laser, and thus PE can serve as the second dye together with fluorescein in dual-label staining.

The source of the various phycobiliproteins leads to considerable variations in the excitation spectra, but much less variation in the emission spectra (see Table

Table 31.1. Some common sources of phycobiliproteins

Source	Phycobiliproteins
Porphyridium cruentum	B-Phycoerythrin, R-phycocyanin
Spirulina platensis	C-Phycocyanin, allophycocyanin
Anabaena variabilis	C-Phycocyanin, allophycocyanin
Gastroclonium colterii	R-Phycoerythrin, C-phycocyanin
Neoagardiella	R-Phycoerythrin, R-phycocyanin

Table 31.2. Properties of phycobiliproteins

Protein	Extinction coefficient	Absorbance maxima	Emission maxima	Quantum yield
B-PE	2.4×10^6	545, 565	575	Near 1
R-PE	2.0×10^6	490, 545, 565	580	0.8
C-PC	1.7×10^6	620	650	0.7
R-PC	0.8×10^6	555, 620	635	0.7
APC	0.7×10^6	650	660	0.5

'Extinction coefficient' is the molar extinction coefficient at the absorbance maximum. Absorbance and emission maxima are all in units of nanometers (nm). B-PE, B-phycoerythrin; R-PE, R-phycoerythrin; C-PC, C-phycocyanin; R-PC, R-phycocyanin; APC, allophycocyanin.

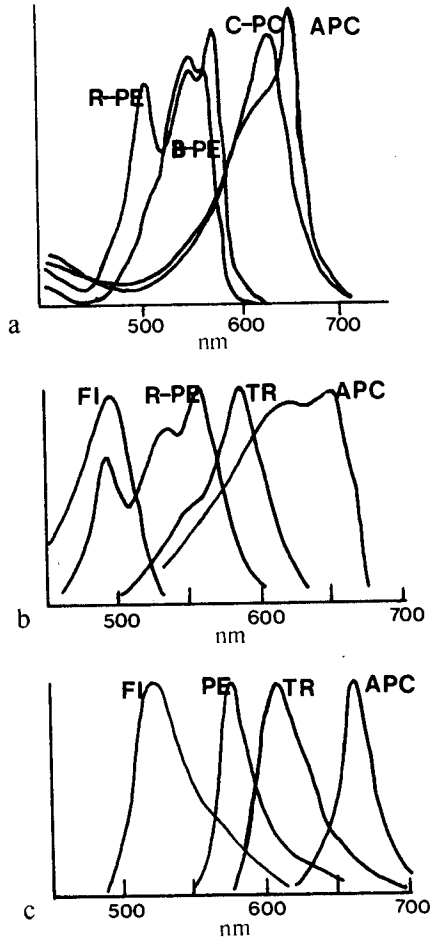

Fig. 31.1. (a) Visible absorption, (b) excitation and (c) emission spectra for fluorochromes employed in flow immunofluorescence analysis.

31.1 and Fig. 31.1). Thus PE from red algae ('R-PE') has about twofold greater excitation at 488 nm compared with PE derived from certain bacterial sources ('B-PE'). Similarly, PC from certain red algae ('R-PC') has considerably greater excitation at 550 nm compared with PC from other algal or bacterial sources ('C-PC'). Therefore, in applications where PE will be excited at 488 nm, R-PE gives about twofold greater signal compared with B-PE; in contrast, for mercury arc excitation (about 550 nm) B-PE gives somewhat greater signal than R-PE.

Isolation and purification

Phycobiliproteins occur at varying concentrations in red algae and cyanobacteria (Table 31.1). As the levels of these proteins are inducible, they are found in greatest yield in organisms growing at relatively low light levels so that yields from harvested red algae vary with the season. Cyanobacteria can be grown in the laboratory under controlled light conditions and provide a stable source of phycobiliproteins. In addition, water extracts of algae often contain large amounts of agar, which complicates the purification procedure. Thus purification from the bacterial sources is preferred, but in cases where R-PE is essential, algae must serve as the source.

Purification of these proteins from either algae or bacteria follows similar steps [12]. As the proteins are water soluble, the first step is dissociation of the cells and preparation of an aqueous extract. Dilute extracts are most conveniently concentrated by precipitation with ammonium sulphate. Chromatography on hydroxyapatite serves to resolve the major pigment proteins; such separation is very apparent because of the distinct colours of the three major pigments (PE, pink; PC, violet; APC, blue). The increasing purity of the proteins can be followed by recording visible spectrograms in the range of 400–700 nm (see Fig. 31.1). A final size fractionation chromatography is usually

employed to eliminate aggregated material and degraded subunits that accumulate to a varying extent during the purification procedure.

Purification of phycobiliproteins from algae

Equipment

Blender (Waring).
Large-volume, high-speed centrifuge (Dupont).
Scanning spectrophotometer (Cary).

Materials

One kilogram of red algae (*Gastroclonium* and *Neoagardiella* work well).
Hydroxyapatite.
AcA 34 gel (LKB).

Procedure

1 Start with at least 1.0 kg wet weight rinsed red algae.
2 Homogenize in a blender using extract buffer to extract water-soluble proteins.
3 Spin down insoluble algal material in large volume rotor (10 000 rev./min for 10 min); collect supernatant containing phycobiliproteins.
4 Re-extract the pellet once (freezing, thawing and homogenizing the pellet may improve the yield).
5 Precipitate phycobiliproteins with ammonium sulphate (35% gives a fraction enriched for APC; 65% gives all phycobiliproteins; add solid ammonium sulphate: 700 g/l is saturated); stir for at least 2 h at 4 °C; spin down precipitate in large-volume, high-speed rotor (Sorvall GSA rotor) at 10 000 rpm for 10 min.
6 Redissolve precipitate in minimum volume hydroxyapatite column buffer; dialyse against this buffer for at least three changes at 1:50 ratio.
7 Pre-equilibrate hydroxyapatite with above buffer (wash with 100 mM-sodium phosphate; then wash with large excess at 1 mM-phosphate; de-fine carefully).
8 Apply dialysed sample to hydroxyapatite column (2.5 × 10 cm) and wash with buffer extensively.
9 Elute PC, PE, APC with increasing concentration of phosphate: steps of 1 mM, 5 mM, 10 mM, 20 mM, etc.
10 Pool PC-, PE- and APC-rich fractions and precipitate with 65% saturated ammonium sulphate (add solid) for at least 2 h at 4 °C; spin down precipitate and resuspend in minimum volume buffer; dialyse against same buffer as described above.
11 Rechromatograph each of the three fractions on separate hydroxyapatite columns (1.5 × 5 cm).
12 Pool and precipitate each pure fraction; redissolve in minimum volume of Tris/saline column buffer; dialyse against this buffer.
13 Chromatograph each of the three fractions on LKB AcA 34 column of appropriate size; collect and pool pure symmetrical protein peak; precipitate with ammonium sulphate, 50% saturation, and redissolve precipitate in a minimum volume of Tris/saline column buffer; dialyse against this buffer. Protein concentration should be about 3–5 mg/ml.
14 Characterize by OD: 1 mg/ml (1 cm cell) gives OD of: 8.2 at 565 nm for R-PE, 7.0 at 650 nm for APC, and 7.6 at 620 nm for PC.
15 Record absorption spectra (400 nm to 700 nm) for a suitable dilution of each fraction (refer to Fig. 31.1).

Notes and recommendations

Phosphate elution point varies from species to species. Slow stepwise increase in phosphate concentration seems to work best (typically 1 mM, 5 mM, 10 mM, 20 mM, 50 mM).

Purification of phycocyanin and allophycocyanin from cyanobacteria

Equipment

Large-volume centrifuge.
Shaking water bath.
Scanning spectrophotometer (Cary).

Materials

Fifty grams wet weight *Spirulina*, pelleted.
Hydroxyapatite.
Lysozyme.

Procedure

1 Resuspend the cell pellet in 500 ml lysozyme extraction buffer.
2 Incubate the suspension at 37 °C overnight in a shaking water bath.
3 Centrifuge the suspension to remove cellular debris.
4 Precipitate the clarified extract with 50% saturated ammonium sulphate.
5 Redissolve the precipitate in hydroxyapatite buffer; dialyse against this buffer extensively.
6 Chromatograph the solution on a hydroxyapatite column (2.5 × 10 cm) to separate phycocyanin from allophycocyanin.
7 Record scanning spectrograms in the 400–700 nm range.

Coupling methods

Several approaches for staining cells with phycobiliproteins have been employed successfully. Perhaps the simplest approach employs the biotin/avidin system [13] in a non-covalent 'sandwich' method: cells stained with biotinated antibody are then labelled with avidin (which has four binding sites per protein) and finally labelled with biotinated phycobiliprotein. This method has the advantage of simplicity in preparation of reagents, together with a minimum of manipulation of the various reagents. The obvious disadvantage is that it requires three-step staining.

Biotin labelling

Equipment

UV/visible spectrophotometer (Zeiss).

Materials

Biotin-*O*-succinimide ester (BIOSEARCH #B-1000).
Antibody purified by affinity or ion exchange.
Phycobiliprotein purified as described above.
Small desalting column (Pharmacia PD-10).

Procedure

1 Equilibrate small (10 ml) G25 column (Pharmacia PD-10) with bicarbonate buffer.
2 Apply sample (1.5 mg/ml; maximum 2 ml) and collect 0.5 ml fractions; pool protein.
3 Adjust protein concentration to 1 mg/ml in bicarbonate buffer.
4 Dissolve 1 mg biotin ester in 1 ml DMSO just before use.
5 Add biotin solution to give a biotin/protein ratio of 75 μg/mg.
6 Mix immediately and rotate at room temperature for 4 h.
7 Re-equilibrate PD-10 column with 50 mM-Tris/saline; pH 8.0.
8 Apply reaction mixture to PD-10 column and collect 0.5 ml fractions.
9 Pool protein fractions (OD at 280 for antibody; OD at absorbance maximum for phycobiliprotein) and add azide to 0.1%.

Avidin/biotin sandwich

Equipment

Air-driven miniature ultracentrifuge (Beckman Airfuge).
Plate centrifuge.

Materials

Biotin-labelled antibody (Bi-Ab).
Avidin.
Biotin-labelled phycobiliprotein (Bi-PbP).
Staining medium.

Procedure

1 All staining should be carried out in 96-well, round-bottom polyvinylchloride plate.
2 Cells should be at a concentration of 0.01–1.0 million in 20 μl.
3 Dilute Bi-Ab such that titred amount of protein is in 25 μl (typically 0.2–1 μg per million cells); immediately prior to dilution, centrifuge stain reagents in Beckman Airfuge at 100 000 ***g*** for 10 min (deaggregates).
4 Aliquot the diluted Bi-Ab into wells following protocol sheet.
5 Add cells to each well; incubate for 15–30 min on ice; incubation time may vary with cell concentration and antibody affinity.
6 Add 100 μl staining medium and pellet cells using centrifuge equipped with plate holders (200 ***g***).
7 Aspirate supernatant; resuspend cells; add 150 μl staining medium; pellet again.
8 Repeat once more; then add 0.5 μg of avidin in 25 μl of staining medium; incubate for a further 15 min on ice.
9 Add 100 μl of staining medium and pellet cells as before; repeat step 7; then add a titred amount of Bi-PbP in 25 μl of staining medium; incubate for 15 min on ice and wash, following above procedure.
10 After last wash, resuspend cells in staining medium and analyse.

Covalent coupling methods

An alternative to the non-covalent staining procedure described above is the use of covalently cross-linked phycobiliprotein–antibody complexes. Several methods have been described for the production of such complexes; most of these are adaptations of procedures originally described for preparation of enzyme–antibody complexes (used in ELISA assays). Two procedures in general use, which have been found to work in most cases, are described below. One, using SPDP [14], results in a disulphide linkage while the other, utilizing SMPB (or its sulphonate derivative), results in a thioether bond [15]. The reactions are summarized in Fig. 31.2.

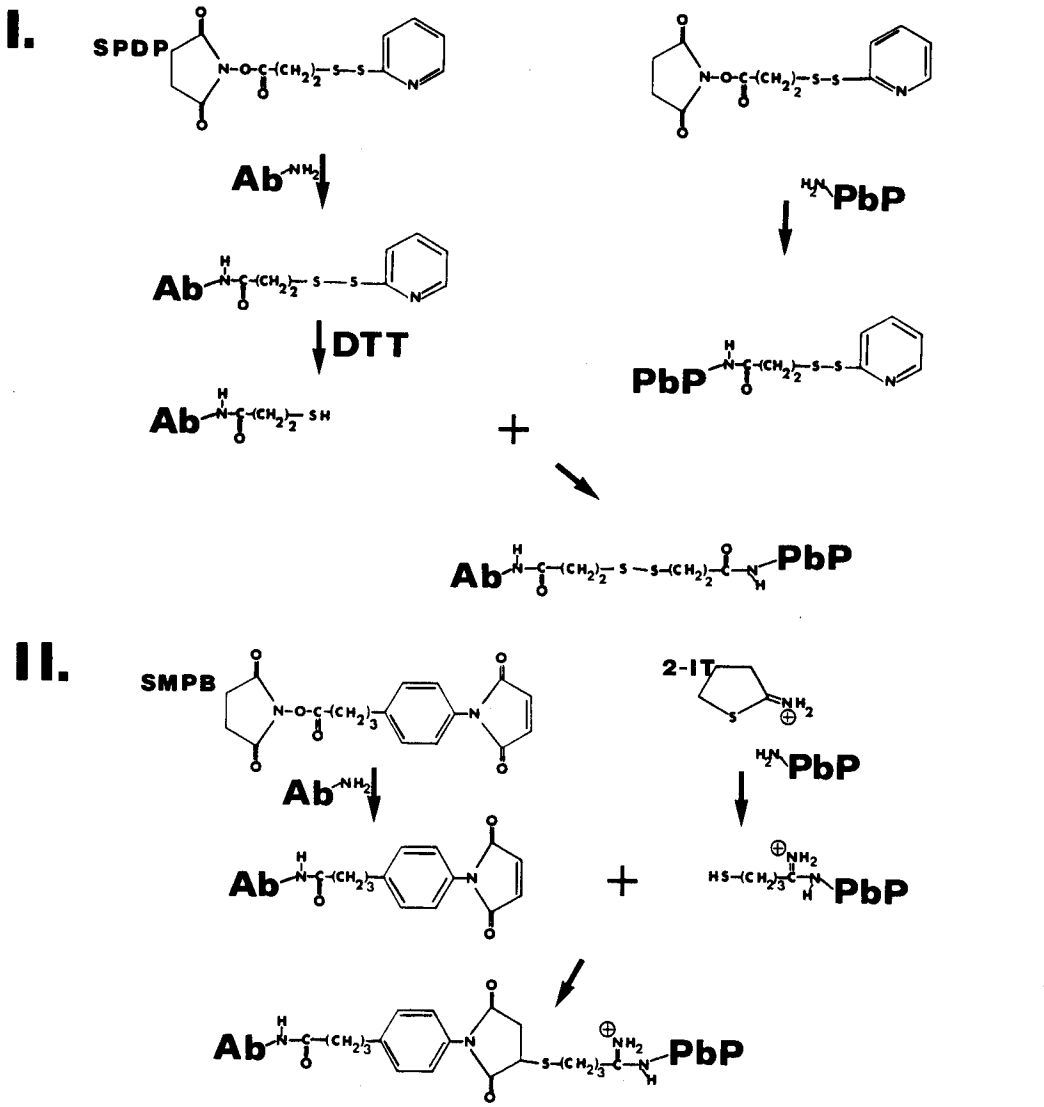

Fig. 31.2. Summary of the (a) SPDP and (b) SMPB labelling reactions for producing antibody–phycobiliprotein conjugates.

SPDP cross-linking

Equipment

Small rotator (Labquake).
UV/visible spectrophotometer (Zeiss).

Materials

Phycobiliprotein.
Purified antibody.
N-Succimidyl 3-(2-pyridylthio) propionate (SPDP), (Mr 312.4), moisture sensitive; refrigerate, Pierce Chemical Co. #21557.
Dithiothreitol (DTT).
Small desalting columns (Pharmacia PD-10).

Procedure

Carry out procedures (i) and (ii) simultaneously. •

(i) SPDP labelling

1 Exchange 1.0 mg PE (in 0.3 ml) into coupling buffer with PD-10 column (pre-equilibrated with coupling buffer), final volume 0.5 ml.
2 Add 50 μl of a freshly made solution of 3 mg SPDP in 0.3 ml methanol to the PE solution; rotate for 1 h at room temperature.
3 Isolate labelled PE with PD-10 column (pre-equilibrated with coupling buffer).

(ii) Labelling of antibody with SPDP

1 Exchange antibody into coupling buffer with PD-10 column (pre-equilibrated with coupling buffer): 1 mg of purified antibody in 0.3 ml.
2 Add 8 μl of a freshly made solution of 3 mg SPDP in 0.5 ml methanol to the antibody solution.
3 Rotate for 1 h at room temperature; isolate labelled antibody with PD-10 column pre-equilibrated with acetate reducing buffer.
4 Reduce labelled antibody with dithiothreitol (DTT) by adding a sufficient amount of 1 M stock solution to give a 50 mM concentration; incubate for 15 min at room temperature.
5 Remove the DTT by dialysis against coupling buffer or by passage down a PD-10 column pre-equilibrated with coupling buffer.

(iii) Conjugate formation and isolation

1 Mix the labelled PE with the reduced, labelled antibody; rotate for 5–8 h.
2 Quench residual thiol groups with iodoacetamide (Mr 185; add solid) to give a final concentration of 30 mM; incubate for 15 min at room temperature.
3 Isolate protein by passing mixture down PD-10 column (equilibrated with appropriate buffer).

Notes and recommendations

It is possible to quantify the degree of SPDP substitution by monitoring the absorbance increase at 343 nm due to release of 2-pyridinethione on treatment with DTT. This procedure requires relatively large quantities of protein (1 mg), which is probably not reasonable for PE, but may be for antibody. The point of the cross-linking procedure is to label both phycobiliprotein and antibody with an average of one SPDP, so that titring the amount of reagent on each protein to achieve this value is the best approach.

SMPB/2-IT cross-linking of phycobiliprotein to antibody

Equipment

Small rotator (Labquake).
UV/visible spectrophotometer (Zeiss).

Materials

5-Iminothiolane hydrochloride salt (Mr 137.6), air and moisture sensitive; refrigerate, Pierce Chemical Co. #26101.
Succinimidyl 4-(*p*-maleimidophenyl)butyrate (Mr 356.32), light and moisture sensitive; refrigerate, Pierce #22315.
Sulphosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (Mr 458.36), Pierce #22317.

Procedure

Carry out procedures (i) and (ii) simultaneously.

(i) Thiolation of PE

1 Exchange 1.0 mg PE (in 0.3 ml) into coupling buffer with PD-10 column (pre-equilibrated with coupling buffer); final volume 0.5 ml.
2 Add 50 μl of a freshly made solution of 3 mg 2-iminothiolane in 0.3 ml coupling buffer to the PE solution; rotate for 1 h at room temperature.
3 Isolate thiolated PE with PD-10 column (pre-equilibrated with coupling buffer).

(ii) Labelling of antibody with SMPB

1 Exchange antibody into coupling buffer with PD-10 column (pre-equilibrated with coupling buffer): 1 mg of purified antibody in 0.3 ml.
2 Add 8 μl of a freshly made solution of 2.5 mg SMPB in 0.1 ml dimethylformamide (DMF) with rapid mixing (otherwise forms rapid precipitate) to antibody solution.
3 Rotate for 2 h at room temperature; isolate labelled antibody with PD-10 column (pre-equilibrated with coupling buffer).
4 Alternatively, use the water-soluble sulpho-SMPB in an analogous fashion.

(iii) Conjugate formation and isolation

1 Mix the thiolated PE with the SMPB-labelled antibody; rotate for 5–8 h.
2 Quench SMPB by adding 1/100 volume of a 1 M solution of dithiothreitol (Mr 154, in coupling buffer); incubate for 30 min at room temperature.

3 Block all sulphydryl groups by adding iodoacetamide (Mr 185; add solid) to give a final concentration of 30 mM (destroys all remaining DTE also); incubate for 15 min at room temperature.
4 Isolate protein by passing mixture down PD-10 column (equilibrated with appropriate buffer).

Notes and recommendations

Other proteins such as avidin or peanut agglutinin (PNA) can be conjugated exactly as described for antibody in the above procedure. As with SPDP conjugations, titring both the 5-IT labelling of phycobiliprotein and the SMPB labelling of antibody to achieve about one label per protein is recommended. Reaction rates are dependent on several factors, including protein concentration and the number of accessible reactive lysines, so some variation is likely to be encountered.

Separation and characterization methods

After production of covalent phycobiliprotein–antibody complexes, the next major problem is the fractionation of the resulting mixture. This step may not be absolutely necessary depending on the coupling procedure, the individual antibody, the ratio of reactants and the application in mind. However, for many applications, the use of a reasonably well-defined reagent is preferable and so fractionation of the coupling mixture is in order.

Two methods are described below that have been routinely employed in the preparation of phycobiliprotein staining reagents. The first is a size fractionation making use of the fact that both antibody (150 kDa) and phycobiliprotein (100–250 kDa) will be included in Sephacryl 300 gel whereas the complexes will move near the void volume [16]. If this fractionation is done carefully, very large aggregates (which may produce much of the non-specific staining background) can be eliminated in the void volume. The other method employs hydroxyapatite chromatography and has the advantages of smaller scale, more rapid fractionation and less dilution compared with gel filtration. One disadvantage of this latter method is that the molecular weights of the fractionated components are not determined.

Fractionation by gel filtration

Equipment

UV column monitor.
Fraction collector.

Materials

Sephacryl S300.

Procedure

1 Pour a Sephacryl S300 column of appropriate size for the amount of conjugate to be fractionated.
2 Wash the column with Tris/saline (pH 8.0), and then apply the conjugate in a minimum volume.
3 Develop the column with Tris/saline; the void volume contains very large aggregates; collect conjugate in the 600–300 kDa range; free antibody and phycobiliprotein elute last.

Fractionation by hydroxyapatite chromatography

Equipment

One millilitre (tuberculin type) syringe barrel.

Materials

Hydroxyapitite.
Small desalting column (Pharmacia PD-10).

Procedure

1 Pour 1 ml column of hydroxyapatite previously equilibrated with 1 mM-phosphate, 100 mM-NaCl, pH 6.8 buffer. A 1 ml syringe barrel works well as a column.
2 Transfer conjugate mixture into this buffer either by dialysis or by passage down a small desalting column.
3 Apply the conjugate mixture to the column; all should bind near the top of the column.
4 Develop the column with a stepwise phosphate gradient; for PE conjugates, PE elutes early (10–30 mM) and conjugates elute later (50–100 mM).

Notes and recommendations

Typically the most tenaciously bound coloured fraction will be the largest aggregate.

Characterization of size, staining and background staining

Equipment

HPLC or FPLC set-up.
Fluorescence microscope.
Flow cytometer.

Materials

Target cells.

Procedure

1 Analyse conjugate fractions by size permeation HPLC to determine the approximate composition of the different fractions.
2 Analyse the fractions by staining appropriate target cells with dilutions of the different fractions.

Notes and recommendations

Experience has shown that the smallest conjugates (less than 10^6 Da) stain adequately with very little background, whereas the larger conjugates (greater than 10^6 Da) often stain much more intensely, but with some background. Even 'good' reagents may pellet in the air-driven micro-ultracentrifuge ('Airfuge') at 100 000 ***g*** in 10 min; accordingly, reagents are routinely deaggregated in a high-speed microcentrifuge (Eppendorf) for 15 min just before use.

Applications of phycobiliprotein reagents

Several applications of phycobiliproteins as fluorescent probes have been published recently [17,18]. In the following sections, some of these applications in flow cytometry are described. In addition, it should be noted that these dye proteins offer possibilities in terms of optimal excitation with a krypton (568 nm, 647 nm) or helium-neon laser (632 nm) or a mercury arc lamp (for fluorescence microscopy and certain flow analysers).

Correlated two-colour staining with one-laser excitation

As was described at the beginning of this chapter, dual-staining in flow cytometry has presented a real challenge, one which has not been met adequately by the fluorescein/rhodamine combination. Dual-laser systems, especially in combination with a dye laser, give high quality correlated immunofluorescence data with fluorescein and Texas red. Unfortunately, this is achieved at the cost of optical and electronic complexity and at considerable added expense compared to single-laser instruments.

It is possible to use PE together with fluorescein on a single-laser machine [7]. The broad excitation spectrum, large extinction coefficient, and high quantum yield of PE make it a reasonable dye to use in combination with fluorescein at 488 nm excitation. As shown by the diagram in Fig. 31.3, the fluorescences are divided by a 560 nm long-pass dichroic reflector, suitably filtered, and then the analogue signals are compensated to correct for the small overlap of PE in the fluorescein channel and the somewhat larger overlap of fluorescein in the PE channel. Data quality is quite good, as exemplified by the two-colour contour plot in Fig. 31.3, and compares favourably with two-colour staining carried out on a dual-laser system.

Three- or four-colour staining with a dual-laser set-up

Phycobiliproteins offer the possibility of carrying out three- or four-colour correlated immunofluorescence measurements [19–21]. Fluorescein and phycoerythrin are excited by the 488 nm laser and compensated as described in the previous section. Texas red and allophycocyanin are excited by a tunable dye laser operating at 603 nm, split by a long-pass dichroic reflector at 650 nm, filtered further and then compensated for the somewhat greater overlaps in the TR/APC fluorescence emissions (compared with the F1/PE combination).

The basic design of the detection set-up is diagrammed in Fig. 31.4. As shown, a half-mirror splits the fluorescence signals from the two-laser spots, dichroic mirrors split these two signals further into the two pairs (F1/PE and TR/APC) that will be compensated and detected as the four individual immunofluorescence measurements. This design permits either four immunofluorescence measurements or three immunofluorescence measurements, together with propidium iodide dead cell gating [22].

Three- and four-colour immunofluorescence open up a new era in correlated flow cytometry (FACS) analysis. Very small populations of functionally distinct lymphoid cells can be discriminated on the basis of the correlated expression of three or four cell surface antigens. In addition, it is possible to include control staining reagents, together with specific reagents, and so determine specific staining on a cell-by-cell basis. An example of this sort of analysis is shown in Fig. 31.5.

Increased sensitivity with reduced background autofluorescence

It is clear that PE provides two- to fourfold greater signal intensity compared to fluorescein at 488 nm. In addition, cell autofluorescence is somewhat lower in the PE channel because autofluorescence is less at longer wavelengths. Thus, although Texas red, phycocyanin and allophycocyanin all generate less absolute signal than fluorescein, separation between stained and unstained populations is often greater compared with similar fluorescein reagents. This decrease in

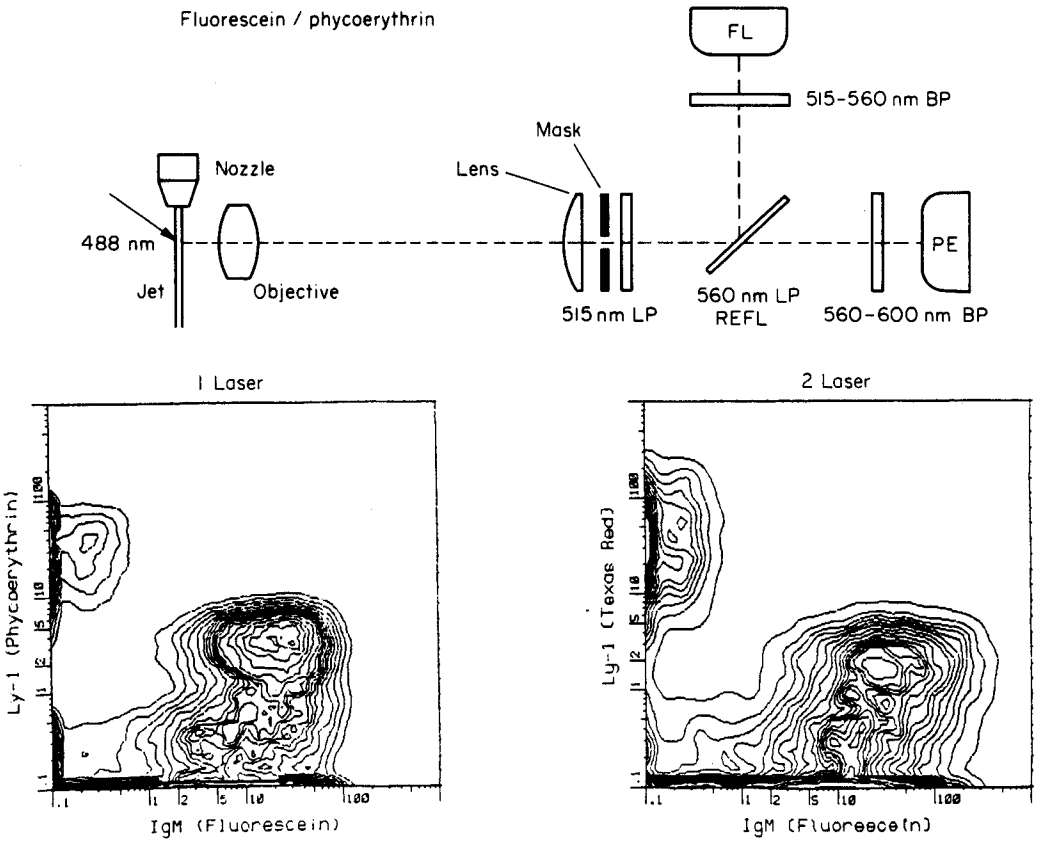

Fig. 31.3. Diagram of a FACS dual-fluorescence/single-laser detection system; contour plot displays compare typical fluorescein/phycoerythrin data with typical fluorescein/Texas red data on peritoneal wash-out cells stained with F1-anti-IgM and PE- or TR- anti-Ly-1. In both data sets, macrophages are eliminated by forward- and wide-angle light scatter gating.

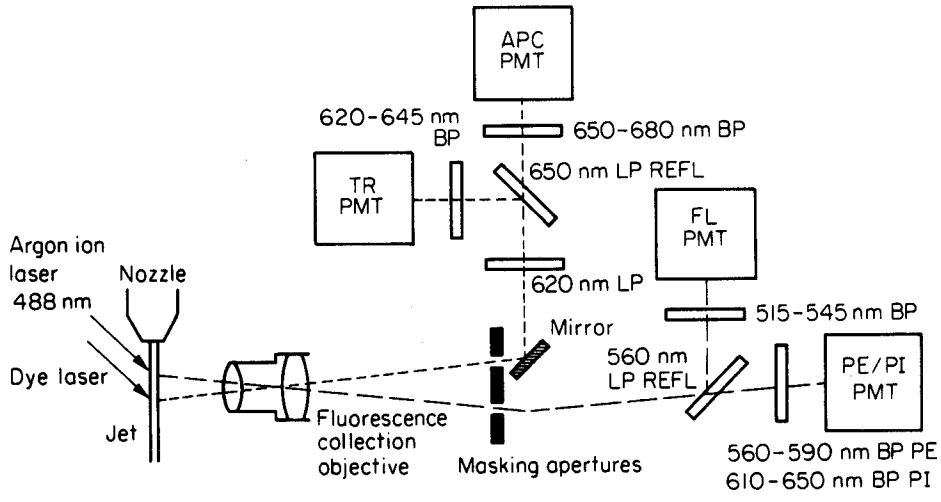

Fig. 31.4. Diagram of a four-fluorescence/dual-laser detection system. The PE/PI detector can be used to measure either fluorescence, simply by changing the final filter in front of that detector.

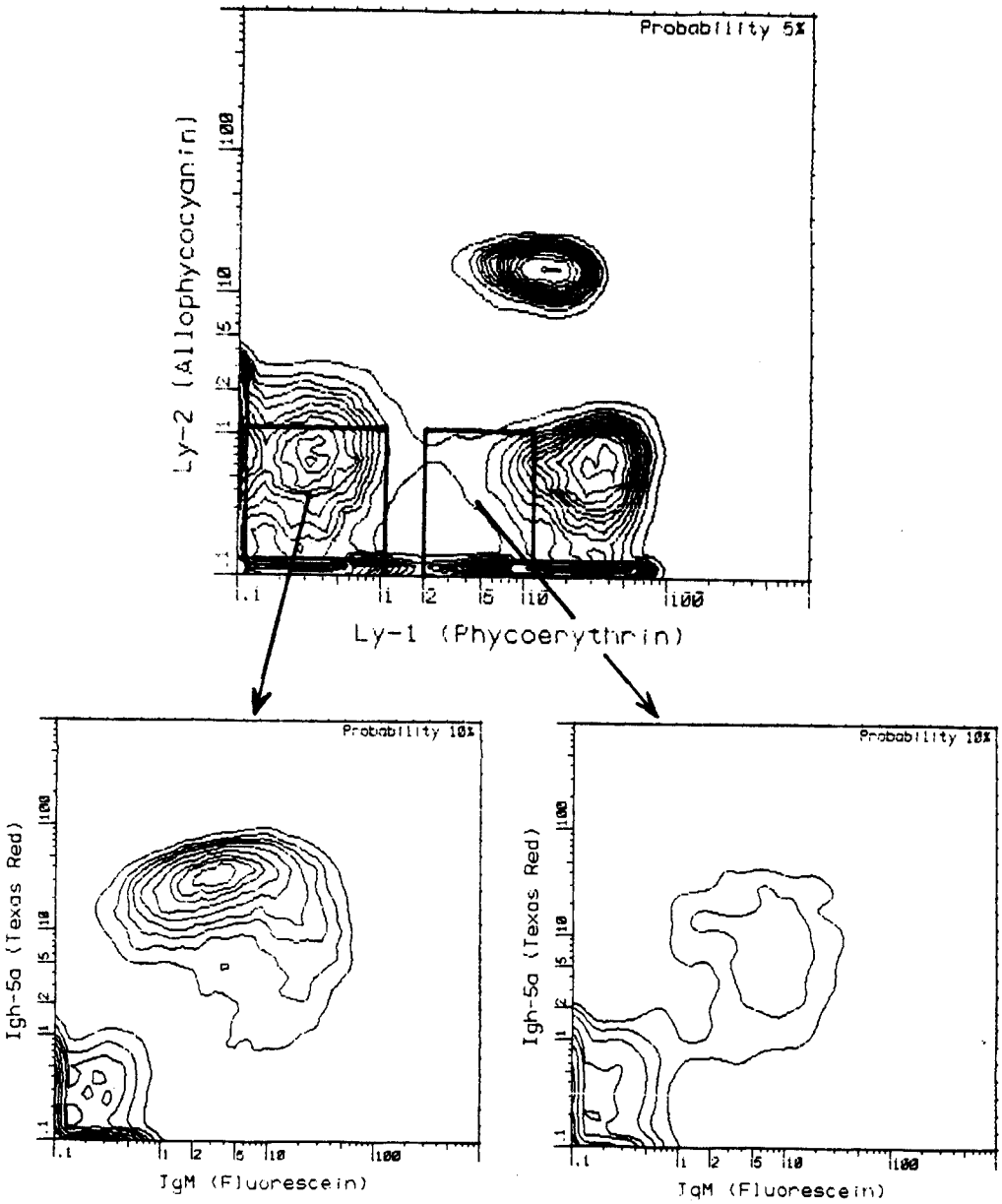

Fig. 31.5. Contour displays of correlated four-immunofluorescence data. BALB/c spleen cells stained simultaneously with F1-anti-IgM, Tr-anti-IgD (Igh-5a), PE-anti-Ly-1 and APC-anti-Lyt-2. The large plot shows the correlated Ly-1/Lyt-2 display. The two smaller plots show IgM/IgD displays for Ly-1$^-$, Lyt-2$^-$ cells (showing 'normal B cells') and Ly-1$^+$ Lyt-2$^-$ cells (showing 'Ly-1 B cells'). Anti-Lyt-2 is the same rat isotype as anti-Ly-1 and so serves as an isotype control to define specific Ly-1 staining on a rare population of B cells. The Ly-1 B cells (IgM$^+$,IgD$^+$) constitute only 2% of the 100 000 cells analysed.

autofluorescence can become very significant when working with weakly staining reagents on cultured cells, which are usually very autofluorescent. Thus the application of phycobiliprotein reagents increases substantially our ability to detect cell surface antigens present at very low (previously undetectable) levels, especially if such antigens are expressed on cultured cell lines.

Buffers and media

Algae extraction buffer

50 mM-sodium phosphate
1 mM-NaN_3
pH 7.0

Lysozyme extraction buffer

0.1 M-sodium phosphate buffer
10 mM-EDTA
pH 7.0
Containing 100 μg/ml of lysozyme

Saturated ammonium sulphate

Heat 1 kg ammonium sulphate in one litre total volume distilled H_2O in a 60 °C water bath for 2–4 h. Remove to a refrigerator or cold room and store at 4 °C for at least 24 h before use.

Hydroxyapatite column running buffer

1.0 mM-sodium phosphate
0.1 M-NaCl
1 mM-NaN_3
pH 7.0

Gradient buffer

100 mM-sodium phosphate.
pH 7.0
Dilute to suitable concentration, make 0.1 M in NaCl, 1 mM in NaN_3

Tris/saline gel filtration column buffer

50 mM-Tris-hydroxyaminomethane (Trisma base, Sigma)
150 mM-NaCl
0.1% NaN_3
pH 8.0

Biotin coupling buffer

0.1 M-bicarbonate
pH should be about 8.4

SPDP coupling buffer

100 mM-sodium phosphate, 35.8 g/l of dibasic ($12*H_2O$)
100 mM-sodium chloride, 5.8 g/l
pH 7.5

Acetate reducing buffer

50 mM-sodium acetate
100 mM-sodium chloride
pH 4.5

SMPB coupling buffer

100 mM-sodium phosphate, 35.8 g/l of dibasic ($12*H_2O$)
50 mM-sodium chloride, 2.9 g/l
pH 6.8

Fluorescence staining medium

Deficient RPMI 1640 (without phenol red, biotin, and riboflavin) containing 0.1% NaN_3, 3% filtered newborn calf serum and 10 mM-HEPES, pH 7.4.

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