

Immunophenotyping procedures can be very sophisticated and provide a large amount of information. However, as with most biological techniques, the preanalytical steps preceding immunophenotyping are of major importance. Here are the consensual considerations that emerged from the group as recommendations for handling samples prior to flow cytometry analysis.

1) Sampling for Acute Leukemia diagnosis

a) **Bone marrow** is preferred, but peripheral blood samples with large blast counts (>80% or >30 G/L) can also be used. Peripheral blood can be used in those cases where it is impossible to obtain an adequate bone marrow sample, i.e. in case of fibrosis. In some cases, the presence of blast cells in a peripheral blood sample may appear as an alarm from an automated haematology analyzer, and this sample can be used for further identification of this abnormal population. A blood film should however be obtained before proceeding with the immunophenotyping, and it must be verified whether this patient is known and a bone marrow sample has already been performed or is planned shortly. There may be some slight differences related to the passage of the blasts towards the periphery, but these should not affect the major markers of lineage assignment, classification and prognosis. In AML cases with undifferentiated and differentiated blast populations the undifferentiated population may be more numerous in the bone marrow than in peripheral blood.

b) The **volume** of the sample collected is also very important. As collection proceeds, the aspiration will progressively empty the bone marrow compartment sampled, which will be replaced by peripheral blood, resulting in a "dilution" of the bone marrow. The latter can be suspected when the number of neutrophils and or the number of normal T lymphocytes is high in the sample (1,2). It could therefore be useful to have simultaneously the number of circulating neutrophils. Iliac puncture is better than sternal puncture in that matter, as it allows for a deeper puncture, and for continued collection over a longer way while slowly removing the needle.

Basically,

- i) the first 0.3 mL should be reserved for freshly "bedside" prepared smears (or granules crushing) and for direct preparation for classical cytogenetics
- ii) the next mL should then be reserved for flow cytometry. At any rate, about 2 mL and no more than 5 mL should be collected in one spot.
- iii) other blast-rich samples are needed for other techniques (molecular studies, storage), and they should be obtained as a new sample collected at the same time, which is possible if the patient is anaesthetised, or planned another day.

Note: If only one sample is available, for instance if it has been sent from elsewhere, the tube provided should be used first to prepare smears (if not provided by the sender), then for flow cytometry and other techniques if enough material is available. If several tubes have been forwarded, one may consider to check their blast count and select the appropriate one for flow cytometry, or to mix all the samples in order to perform the different techniques on a homogeneous preparation.

- c) As **anticoagulant**, both EDTA, Heparin or Na citrate are suitable.

i) EDTA/K is better used at a concentration of 2 mg/mL Na₂EDTA (3). EDTA reduces leukocytes and platelets adhesivity and aggregation but damages cells within 24h: it is best for analysing samples within 2-6 h.

ii) Heparin (200 U/mL) may interfere with morphology, labelling, fixation, permeabilization and Taq polymerase although it is the preferred anticoagulant for classical cytogenetics (4,5). Using heparin, samples are stable for 48h, and it thus could be considered the best anticoagulant for those samples that will be analyzed with a known delay (i.e. transportation).

iii) With ACD cells viability is very high within 72h maintaining samples at room temperature (18-22°): it seems to be the best recommended anticoagulant according to the literature (6).

Note. it is important to emphasize for the personnel in charge of sample collection that proper mixing of the bone marrow and the anticoagulant is mandatory as bone marrow is highly likely to clot rapidly. The generalization of sampling in vacuum-tubes has resulted in the disappearance of the habit of slowly homogenising the sample and the anticoagulant by turning the tube upside down several times.

2) Sampling for CLL or NHL diagnosis

a) Peripheral blood is usually tested in these cases, with the same anticoagulant, EDTA-K however being the preferred choice. There is less risk of clotting for such samples, which are collected by routine phlebotomy.

3) Transportation

Two cases must be distinguished, first from the ward to the laboratory in the same hospital, second from hospital to hospital

a) In both cases, great care should be taken about the **temperature** at which to keep the sample. Room temperature, i.e. between 10 and 25 °C is ideal. The sample should not be refrigerated and certainly not be frozen, it should not stay in the sun either.

b) For samples carried between hospitals over long distances, a special container stabilizing the temperature should be used, and the sample shipped as quickly as possible using a special transporter. If flight transport is used the sample should be kept in the cabin.

c) The **time schedule** to respect is also important. The ideal is "as soon as possible" after collection. In any case, there should be no more than 24-48 hours between collection and sample processing. If a longer delay is expected, one alternative is to separate the mononuclear cells containing the blasts by gradient centrifugation on ficoll and freeze the cells. Another alternative is to delay sampling until the next working day. Again, if for some reason the sample cannot be processed immediately (i.e. arrival in the laboratory late afternoon), it should be maintained at room temperature. Careful resuspension of the samples will be mandatory if there is any delay.

4) Culture medium

a) Although leukemic cells can be metabolically very active and growing, there is usually no need to supplement the sample with culture medium. If preservation appears needed, McCoy's (7) or Terasaki medium are specific for this purpose. The use of phenol red should be avoided as it may interfere with fluorescence, or select cells.

b) No stabilizer should be used. Their effects on leukemic cells are not well documented although they are known to modify light scattering positions of clusters, impairing a proper discrimination of abnormal and normal cells, and may destroy or critically change antigen expression.

5) Enumeration

Blast cell enumeration should ideally be performed specifically on the sample that will be immunophenotyped. Haematology analysers are well suited to this. It may be useful to compare the results obtained in the laboratory to external data from the patient.

6) Morphology

Again, this should also be checked on the sample that will be immunophenotyped, if it is specific. This is important with regard to hemodilution, but also for samples that have travelled, together with properly prepared fresh smears or cytopins, to check the integrity of the cells and absence of selection. Hemodilution may also be considered in such cases. A high proportion of smudge cells (Gumprecht shadows) leads to discrepancies between cytology and flow.

7) Sample handling

Although all immunophenotyping laboratories should have their validated procedures (SOP), here are some consensual remarks about sample preparation, differentiated for whole and separated samples.

a) Whole blood/ whole bone marrow

i) Dilutions/adjustment

(1) Adjust the cell concentration to <10 million/mL if there is a high leukocyte count

(2) On this basis, or for lower counts, adjust the volume to the panel of antibodies to be tested, with 50µL of sample per tube

(3) Consider storing extra cells in rich samples

(4) Use antibodies in excess amounts, determining the right concentration by previous titration.

ii) Red blood cells (RBC) lysing reagents. These reagents may induce selective cell loss or impair proper labelling and should be used in very standardized procedures.

(1) NH₄Cl : requires highly standardized and validated procedure in order to avoid cell loss by prolonged contact with the lysing reagent (8-10).

(2) NH₄Cl can be used before labelling

(3) If lysed/washed cells : consider blocking Fc receptors again with Ig preparations (Behring, concentration around 20g/L), human AB serum, BSA or animal serum from the same species as the antibodies used.

- (4) Commercial lysing reagents usually also fix and sometimes tend to permeabilize and should be used after labelling
 - (5) Lyse and wash vs no wash is left to the habits of the laboratories and their validation of the technique chosen. No wash protocols may sometime induce discrepancies by the presence of unbound antibodies or low affinity binding (competition between bound and unbound immunoglobulins).
- iii) Fixation
- (1) Paraformaldehyde 0.1 to 0.5% in Phosphate Buffered Saline (PBS) will fix the cells, impairing the process of patching and capping that may result in the disappearance of the labelling by endocytosis of the surface antibodies by a living cell. Reduction of the metabolic activity of labelled cells can also be achieved by maintaining them at +4°C as soon as they have been in contact with antibodies, and/or adding 1‰ sodium azide in the washing buffer. (the acquisition could be performed without bias the day after). Fixation will maintain the fluorescence over several days, and will also neutralize any virus present in the sample (11-13).
 - (2) Fixation is not necessary if the flow cytometry reading is proceeded with as soon as the labellings are over, but the use of 1‰ sodium, azide in the PBS used for cell suspensions also is good laboratory procedure.
- iv) Cytoplasmic labelling
- (1) For flow cytometry, the sequence of manipulations will be fixation + permeabilization + labelling. The use of ethanol will provide both fixation and permeabilization. Most laboratories use commercial reagents and their specific protocols, or in house 1% saponin preparations (14).
 - (2) A change in cell scatter properties of the cells is to be expected but can be avoided
- b) Cell separation
- i) Ficoll Hypaque is the most commonly used preparation. It was initially devised by Boyum (15) to separate peripheral blood lymphocytes, and thus has the density of these cells, i.e. 1.077. Ficoll will nonetheless allow a proper enrichment in the blastic population for most cases of leukemia, while removing red blood cells and mature polymorphonuclears that are heavier and will drop to the bottom of the tube.
 - ii) Used properly, Ficoll separation does not alter the cells. The separation should be carried out at room temperature, immediately after slowly depositing the diluted sample (1V/1V) over 1V of Ficoll. The centrifugation time and speed should be around 20 mn at 600g, with no brake. It is also important to rapidly collect the cells at the interface and wash them (in a large volume (~15mL) of PBS or better Hank's or RPMI1640 medium), by another centrifugation at 800g. If red blood cells remain, the first wash could be done with NH₄Cl. In some cases, the Ficoll cell separation technique can artificially select cell populations (16) and/or cell loss (17).
- Note* : Other media such as Percoll gradients could occasionally be used.
- c) Morphologic control

- i) A cytocentrifuge smear of the suspension can be prepared and stained for microscopic control.
- ii) The scattergram in flow cytometry will also provide a good impression of the cell suspension's composition.
- iii) Conjugation of both techniques is the most satisfactory

d) Quality control

The most important control, after checking the morphology of the cells in the samples and the blast count, is to perform a **viability check**.

- i) Trypan blue is useful in microscopy. A small aliquote of the cell suspension is mixed with trypan blue (1:2 with 0.4% w/v trypan blue). About 10-20 µL are needed for then being mounted in a haemocytometer (Thoma, Nageotte, Neumayer...). The cells are examined in light microscopy. Blue-stained cells will be the dead cells (18,19).
- ii) Acridine orange/IP can also be used using fluorescence microscopy, as dead cells will appear red and living cell-green. Furthermore, this method identifies apoptotic cells and allows to distinguish between lymphoid cells, monocytes and myeloid cells by the green and orange labelings (20,21).
- iii) Flow cytometry can also be used by using the sensitivity of the light scatter signals.
- iv) Alternately, staining of an aliquote of the cell suspension can be performed with thiazol orange (all nucleated cells are green, stock 1 mg/ml in methanol, final dilution 1:10.000) an Propidium Iodide (stock 1 mg/ml, final 50 µg/ml) (22,23) or 7ADD (all viable cells are negative) or other viability reagents. Although it may be done, it is usually not necessary to add the viability control in all tubes.
- v) One tube with Syto16 or LDS, staining all nucleated cells, could be useful to set up bitmaps.

e) Fixation

- i) See the paragraph above about paraformaldehyde

f) Intracytoplasmic labelling

- i) Flow cytometry (see above)

Cytocentrifuge smears can also be used. Cell fixation and permeabilization can be achieved with cold (+4°C) EtOH, MeOH or acetone. The cells should then be rehydrated in PBS. One antigen only can be tested per smear, but this technique allows for a direct morphological control of stained cells (24).

8) Cryopreservation

For further use in flow cytometry, cells must be preserved as live cells, deep frozen with a cryoprotectant. The most widely used is dimethylsulphoxide (DMSO) at a final concentration of 10%. The important fact to know about DMSO is that it is toxic for cells at room temperature. Great care should therefore be taken during the preparation step, to be sure that everything (cell suspension and media) is cooled at +4°C before mixing the cells and the preservative, and then to be as quick as possible to prepare the aliquots and bring them to a colder temperature (see below). The next critical step, which also requires swiftness and no

waiting time, is that of thawing, until all trace of DMSO has been removed from the cell suspension (25-28).

a) Cryopreservation

- (1) Cell suspension : 10-40 million /mL final in culture medium (RPMI1640 or IMDM)
- (2) 10% final DMSO concentration
- (3) 20% to 80% fetal calf serum (FCS)
- (4) Prepare the same volume of cell suspension at 10 million /mL and 20% DMSO
- (5) Cool separately, until both reach +4°C
- (6) Slowly add DMSO to the cell suspension, while shaking gently to ensure homogenisation
- (7) Quickly transfer 1.5 mL aliquotes to 1.8 mL pre-labelled cryotubes (leave space and respect the upper level as the suspension's volume will increase in the cold)
- (8) Slow freeze at -80°C in special box kept at room temperature \pm isopropanol
- (9) The next day, store the cryotubes in cryoboxes, and store in N₂ vapour or at -80°C

ii) Thawing

- (1) Prepare a 37°C water bath, and the requisite number of tubes with room temperature or 37°C culture medium (RPMI)
 - (2) Quickly thaw one or two aliquotes at a time, directly taking them from N₂ vapour or -80°C to the water bath, with constant vigorous shaking of the cryotubes to ensure permanent contact with warm water (no temperature gradient around the tube)
 - (3) When only a small part of the suspension is still frozen (about the size of a match's head), dry the tube, open it and transfer the cell suspension immediately to the medium previously prepared
 - (4) Immediately centrifuge at room temperature for 10 min at 800g
 - (5) Immediately replace the medium with fresh one
- Notes:* Some very fragile cells may be thawed in 0,5mL of 100% FCS then transferred to fresh medium. It is usually better to allow AML cells to stay in suspension at room temperature for 1-2 hours before labelling.
- (6) Cell viability, as described above, should be checked after thawing.

b) Cell pellets can be stored for further DNA studies. These will not be useable for flow cytometry, while both RNA and DNA can be extracted from viable thawed cells. This procedure may be considered for small remaining amounts of cells.

- i) Adjust to 10-15 million cells per tube
- ii) Pellet cells by centrifugation 10mn at 800g
- iii) Remove medium
- iv) Freeze at -80°C or in N₂ vapour

c) Slides : unstained smears or cytopspins maybe stored for later investigations in immunostaining or FISH.

- i) Air dry the slides
- ii) Wrap them individually in foil and label them

- iii) Store at -80°C for long-term or -20°C for short term storage (up to one month)
- iv) Thawing
 - (1) Bring to room temperature
 - (2) Unwrap
 - (3) Fix

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