Cell Purification: A New Challenge for Biobanks

Maria Almeida Andres C. García-Montero Alberto Orfao

Spanish National DNA Bank Carlos III, University of Salamanca, Salamanca, Spain

Key Words

Biobanks · High-throughput cell sorting · Pure cells · Fluorescence-activated cell sorting · Magnetic-activated cell sorting · High-quality sample collections

Abstract

Performing ‘-omics’ analyses on heterogeneous biological tissue samples, such as blood or bone marrow, can lead to biased or even erroneous results, particularly when the targeted cells and/or molecules are present at relatively low percentages/amounts. In such cases, whole sample analysis will most probably dilute and mask the features of the cell and/or molecules of interest, and this will negatively impact the results and their interpretation. Therefore, frequently it is critically important to have well-characterized and high-quality purified cell populations for the reliable detection of subtle variations in their specific features, such as gene expression profile, protein expression pattern and metabolic status. Biobanks are technological platforms which aim to provide researchers access to a large number of high-quality biological samples and their associated data, particularly to support high-quality scientific and clinical research projects, and such projects will benefit enormously by having access to high-quality purified cell populations or their biological components (e.g. DNA, RNA, proteins). Therefore, a clear opportunity exists for preparative cell sorting techniques in biobanks. Although multiple different cell purification approaches exist or are under development (e.g. cell purifica-

tion techniques based on cell adherence, density and/or cell size properties, methods based on antibody binding as well as new lab-on-a-chip purification techniques), the choice for a specific technology depends on multiple variables, including cell recovery, purity and yield, among others. In addition, most cell purification approaches are not well suited for high-throughput (HT) purification of multiple cell populations coexisting in a sample. Here we review the most (currently) used cell sorting methods that may be applied for sample preparation in biobanks. For the different approaches, technical considerations about their advantages and limitations are highlighted, and the requirements to be met by a HT cell sorting technology to be used in biobanks are also discussed.

© 2015 S. Karger AG, Basel

Introduction

In the last two decades, genomics and proteomics, as well as other ‘-omics’ technologies, have emerged as fundamental tools for the analysis of both genetic and molecular alterations involved in protection and control versus susceptibility for development and progression of many diseases [1–5]. In addition, it is known that there are multiple epigenetic and RNA-associated mechanisms responsible for modulating the expression of a variety of genes, which are therefore also involved in the protection versus the onset and development of many human dis-

eases [6, 7]. For this and other reasons, current research is focused not only on the variability of DNA sequences, but also in the modulation of the expression of such gene sequences through distinct epigenetic and noncoding RNA mechanisms, the assessment of RNA transcription, its translation into proteins, the posttranslational modifications of said proteins, and the interactions among them and between them and other cell components and metabolites [8, 9].

The utility of microarray-based transcriptomic studies and protein expression profiling, for example, has been extensively demonstrated in many different areas, including the evaluation of drug effects on specific cell types, the understanding of the molecular mechanisms and signaling pathways controlling cell growth, proliferation, differentiation and death as well as its alterations (e.g. during carcinogenesis), the identification of different prognostic subclasses among specific diseases, the identification of specific genes whose expression changes in response to pathogens or other organisms, and the discovery of new drug targets and diagnostic markers, among other areas [10–14]. However, many biological samples such as peripheral blood (PB), bone marrow and other body fluids, as well as solid tissues, all consist of admixtures of different cell types, cell lineages and cell maturation/activation states at variable proportions. Because of this, transcriptomic and proteomic analyses, for example, performed on highly purified cell populations are frequently required for reliable detection of subtle variations in their contents, including their specific gene expression pattern, that otherwise could not be identified whenever single-cell-based approaches are not feasible [15]. Otherwise, performing genomic, epigenomic, transcriptomic, proteomic and metabolomic studies on heterogeneous cell samples can lead to biased or even erroneous results whenever the information we are looking for specifically resides in a minor cell population from the whole sample cellularity. In fact, analysis of the whole sample may dilute and mask the specific features of the cell(s) of interest, preventing adequate identification and characterization of molecular mechanisms occurring in the targeted cells whenever these only represent a fraction of all cells in the sample. Consequently, in many circumstances it is critically important to perform such analyses in highly purified cell populations and therefore to have them purified and stored in advance in relatively high amounts and optimal conditions to perform such studies [16–19].

Biobanks are technological platforms which aim to support biomedical research and whose main purpose is to provide researchers access to a large number of high-

quality biological samples and their associated data [20, 21]. Thus, the availability of high-quality cell populations and/or their components purified from biological samples stored in biobanks will tremendously increase the quality of the stored materials, and at the same time will also facilitate and foster their usage by researchers. In fact, the availability of high-quality samples is a major competitive advantage for the advancement of knowledge about health and human disease since it facilitates the discovery of those biological and environmental factors and mechanisms involved in the development, progression and control of specific diseases. Such high-quality standards largely depend on the availability of multiple types of cell populations purified from heterogeneous biological samples. Although the number of biobanks in Spain, Europe and other regions of the world has grown exponentially in the last years, currently there are still very few biobanks which offer their users access to collections of samples which have been fractionated into different, multiple, purified cell compartments and therefore facilitate the development of those projects in which the analysis of purified cell populations is essential to ensure full success of the research performed. This limited availability of purified cell fractions in biobanks is mainly due to the fact that high-throughput (HT) cell purification technologies which can be easily implemented in routine in biobanks are still lacking. This typically leads to the need to evaluate and implement different cell purification techniques and their adaptation for HT purposes prior to selection and incorporation of cell purification technologies into a biobank.

Cell Purification Technologies

Cell purification has proven to be a very powerful tool in biomedical research and even in routine clinical diagnosis and patient treatment whenever isolation of one or multiple cell types (and/or subcellular components) from a heterogeneous cell sample, in the absence of contamination by other cell populations/components from the sample, is required for adequate cell analysis and cell therapy procedures, or as a preparative step for performing further assays [16]. In biomedical research, cell purification has been widely used for many different purposes, including tissue engineering and regenerative medicine, cancer therapy, and research on the pathogenesis of infectious diseases [22–24]. Overall, this is reflected in more than 40,000 publications, many of which include high-impact scientific papers, with a progressive increase in the number of citations per year (fig. 1). From a clinical point of

Fig. 1. Number of citations per year (from January 2000 to September 2013) in which human cell sorting/purification alone (**a**) or together with biobanks (**b**) were included as key words for the manuscript search. Source: Web of Knowledge [v.5.11]-Web of Science Citation Report (<https://apps.webofknowledge.com>). **a** Search key words were as follows: citation report: Topic = (human samples) AND Topic = (cell sorting) OR Topic = (cell purification).

Publication years: from 2000 to 2013. **b** Search key words were as follows: citation report: Topic = (human samples) AND Topic = (cell sorting) OR Topic = (cell purification) AND Topic = (biobanks). Publication years: from 2000 to 2013.

view, usage of highly efficient cell purification techniques has significantly contributed to the diagnosis and treatment of multiple human diseases [17, 25, 26]. Availability of purified cell populations facilitates diagnosis of clonality in the absence of cytogenetic/molecular markers, contributes to increase the knowledge about intratumoral cytogenetic heterogeneity and clonal evolution pathways of different neoplastic as well as nontumoral disorders, and helps in the diagnosis and prognostic assessment of patients with neoplastic (e.g. multiple myeloma and mastocytosis) and nonneoplastic immunological disorders (e.g. primary immunodeficiencies), among other diseases [27–31]. In addition, cell sorting techniques have also been employed extensively for cell therapy purposes, such as transfusion of red cells, platelets and neutrophils, together with transplantation of specific populations of purified hematopoietic and immune cells, including mesenchymal stem cells derived from PB and fat tissue [32–35]. While biomedical research has strongly promoted the development of different cell purification techniques and technologies, clinical applications of cell purification have emphasized the need for highly efficient HT Food and Drug Administration (FDA)/European Medicines Agency (EMA)-approved cell purification devices and standard operating procedures [36].

Because of all the above, a large variety of cell sorting methods and technologies are currently commercially available. The available technologies use multiple distinct principles and features to classify cells. Among others, physicochemical, immunophenotypic and functional properties of the cells of interest have been used to isolate cells [16]. Thus, cells to be purified have been traditionally targeted by four major features: cell adherence properties, density and size, morphological characteristics, and antibody-binding immunophenotypic properties [19]. More recently, new cell purification methods based on microfluidic technologies which take advantage of other cellular characteristics such as cell elasticity in response to acoustic waves, or membrane polarization in a nonuniform electrical field are under development and evaluation [37, 38]. A schematic representation of the different cell isolation/purification techniques based on the above-mentioned properties is shown in figure 2. In addition, a brief comparison of the most widely used and promising cell purification techniques based on cell adherence, density/size, morphological appearance and antibody-binding properties is provided in table 1. As shown there, cell purification methods based on physicochemical features of the cell of interest, such as adherence and density/size, allow the isolation of high numbers of viable

Fig. 2. Schematic representation of different cell isolation/purification techniques, including microfluidic-based technologies, which rely on cell adherence properties, density and size, antibody-binding immunophenotypic properties, morphological and cytochemical features of cells, for their isolation/purification.

Table 1. Relevant features of different cell purification/isolation and/or cell depletion techniques based on the adherence, density/size, morphological appearance and antibody-binding properties of the targeted cells and cell populations

Features of the method	Cell isolation technique						
	adherence	filtration	centrifugation	panning	MACS	FACS	LMD
Principle	adherence	size	density	antibody/size	antibody	antibody	morphological/ cytochemical/antibody
Purity	low	low	low	medium	high	high	high
Recovery	high	high	high	medium	medium	medium	high
Yield	high	high	high	medium	medium	low	low
Efficiency	low	low	low	medium	high	high	low
Cell viability	high	high	high	high	high	high	no
Sample volume	high	high	high	high	medium	medium	small
Throughput (speed)	high	high	high	high	medium	medium	low

cells from large volumes of sample, with both a high yield and throughput (e.g. in terms of time required). However, these methods have important limitations since the purity of the isolated cells is usually low and considered to be insufficient for the purposes that cell purification is frequently requested. In turn, antibody-based purification methods used in combination with cell size properties to isolate the cells of interest, such as cell panning, also permit obtaining viable cells from large volumes of sample in a short time, with a higher purity than that obtained with the cell purification techniques based on cell adherence and/or density/size. However, the cell recovery and yield rates are lower with this latter approach. Other antibody-mediated cell isolation methods such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) are highly efficient cell purification techniques for the isolation of viable cells with a much higher purity, but both methods present several limitations. Since they do not permit working with large volumes of sample, they usually require longer sorting times, and both the cell recovery and yield are lower than those obtained with the former methods. Other relevant cell sorting techniques include laser-based tissue microdissection (LMD), which combines antibody-based cell purification methods with the usage of morphological and cytochemical characteristics of the cells of interest. This approach allows for good recovery of highly purified cells, although it only permits analysis and dissection of small volumes of a tissue; in addition, the efficiency, yield and throughput of this method are low, as well as the viability of the isolated cells. Below we describe in more detail the principles and the most relevant features of each of these cell isolation technologies.

Cell Purification Techniques Based on Cell Adherence Properties

Purification techniques which take advantage of the unique adhesion properties of the cell of interest are rather simple and inexpensive. Because of this, such techniques have been extensively used for the isolation of cells from enzymatically digested, mechanically disaggregated and/or explanted primary tissues [19]. However, in most instances these techniques do not provide high purity because they are based on the adhesion capacity of the cells of interest, which is frequently shared also by other adherent cells in the sample. Although significant progress has been made regarding the variety and properties of the adhesion surfaces used [e.g. adherence of cells to polymer-brush-grafted glass beads [39], cell adhesion on micro-/nanostructured surfaces and ligand-specific (protein, peptide and aptamer) cell adhesion [40]], at present, usage of adhesion-based cell isolation is almost restricted to those applications which do not require high purity or for negative selection of a specific cell population (e.g. depletion of monocytes from PB samples). In addition, since an incubation period (e.g. at cell culture conditions) is required until adherent cells can be selected or depleted, these techniques can also modify the biochemical and molecular properties of the selected adherent cells [19].

Cell Purification Techniques Based on Cell Density and/or Size

Several methods are currently used for the isolation of specific cell populations which rely on the differential physicochemical characteristics of distinct cell populations coexisting in a sample. Thus, most frequently the

unique density and/or size of the cells of interest is used for their purification. Such cell isolation techniques are commonly used for fractionation of tissues containing heterogeneous cell mixtures, particularly for body fluids such as PB, bone marrow and fat tissue [19]. Overall, these are 'bulk' sorting methods that allow isolation of large numbers of cells in a relatively short period of time. Among these techniques, the most extensively used are based on cell density properties that can be revealed by centrifugation; however, cell sedimentation (density-based) and filtration (size-based) methods have also been extensively employed [41, 42]. In practice, density-gradient centrifugation techniques are mostly used for the isolation of different types of cells from PB and blood-containing samples, as well as to exclude dying cells from other tissue samples. For this purpose, the sample is placed on one or more layers having distinct densities, which are intermediate between those of the cells that are to be isolated and all other cells in the sample. Then, centrifugation of the sample at the appropriate speed (e.g. 600 g) fractionates it into distinct phases between the different density layers. For the isolation of PB mononuclear cells (MNC), these typically include the plasma (which contains platelets if they have not been already removed by a prior low-speed centrifugation step), an interphase containing the MNC, the intermediate-density exogenous fluid, and the lower phase containing both the erythrocytes and the neutrophils. Other cell fractions can be further isolated from the MNC fraction through further centrifugation steps where MNC are placed over distinct density-gradient fluids and/or when distinct angular speeds are used specifically devoted to the isolation of the cells of interest. Despite large-scale usage of density-based cell isolation techniques, their specificity is low for the purification of specific and well-defined subtypes of MNC. Because of this, these approaches are mostly used for the isolation of red blood cells and platelets. In addition, they are also used to obtain MNC from large volumes of sample, when specific isolation of distinct subtypes of MNC is not required (e.g. apheresis procedures), or as a pre-enrichment step to remove unwanted cells (e.g. erythrocytes, neutrophils, platelets and dead cells) [19].

Another density-based method which is also widely used for the isolation of specific subpopulations of MNC from blood-containing samples is based on antibody-mediated erythrocyte rosetting. This method relies on the combination between antibody binding and density-based cell purification methods. Briefly, undesired cells are specifically labeled with antibodies that subsequently

form complexes with erythrocytes; thus, they generate immunorosettes of higher density than that of the cells of interest. After centrifugation of the sample, the immunorosettes containing undesired cells are pelleted with the erythrocytes coexisting in the sample, allowing the isolation of the target MNC at the MNC interphase after density-gradient centrifugation [43]. These techniques can also be used for positive selection of erythrocyte-rosetting cells; in such cases, further erythrocyte-lysing procedures are required for final purification of the pelleted cells of interest.

Regarding filtration techniques, isolation of target cells is based on their unique size-associated features. Of note, these techniques are relatively simple methods which are generally employed for cell enrichment as a preparative tool for further cell purification steps [42]. This is due to the fact that filtration techniques are useful approaches for the removal of debris, dead cells and cell aggregates, particularly during the preparation of single cell suspensions from solid tissues. Depending on the specific cells and/or cellular components to be isolated, filters with different pore sizes and which are built of distinct materials are used [44–47]. Additionally, these methods can also be applied for the isolation of large-size cells [16]. However, filtration techniques are usually associated with poor recovery rates due to significant cell loss during the process of filtration. Depending on the cells to be selected or removed, filters with different pore sizes and properties may be used.

Of note, other cell sorting techniques exist that combine usage of both cell size and density features. Thus, centrifugal elutriation has been successfully used for cell purification purposes, which allows a high recovery of viable cells with relatively low cross-contamination by unwanted cells in a single-step procedure [48–51]. Through this cell isolation method, cells are targeted through their unique rate of sedimentation, separation being dependent on both cell size and the difference between the densities of the distinct cells in the sample and the selected cell isolation medium [52].

Cell Purification Techniques Based on Antibody Binding

Currently, antibody-mediated cell isolation methods are the most widely used approaches for the purification of individual cell populations with high purity. For this purpose different techniques are commonly used, which mainly include FACS and MACS [53, 54]; however, other traditional antibody-binding-based cell purification techniques (e.g. cell panning) can also be used. Although

both panning and magnetic cell sorting techniques (MACS) are bulk methods that allow rapid isolation of large numbers of cells, they can only operate according to the selection of a single (or at maximum a few) cell surface marker in a 'yes or no' decision manner, being typically unable to differentiate between cells expressing low versus high levels of a given target marker on their surface membrane [55]. In addition, other cell purification methods that combine antibody-based cell isolation with the usage of morphological and cytochemical characteristics of the cells of interest have been developed to purify specific cells from tissues, such as LMD [56].

Cell Panning Techniques

Cell panning techniques consist of a rather simple approach for the specific isolation or depletion of one or more populations of cells from heterogeneous cell samples. It is based on the principle that antibody molecules adsorbed onto polystyrene surfaces specifically bind to antigens present on the surface membrane of the cells to be selected or depleted from the sample [57]. For these purposes, polystyrene dishes are coated with antibodies specific for one or multiple cell-surface antigens, allowing the targeted cells to bind to the surface of said dishes. Of note, the binding surfaces may have different shapes, e.g. Petri dishes, microbead particles, polystyrene wells and tubes. Overall, this method permits the recovery of isolated cells with a high yield and relatively low levels of contamination by other unwanted cell types; at the same time it most frequently allows the isolated cells to retain their viability and functionality [58]. Another advantage of the method is that fast cell isolation procedures can be performed under sterile and temperature-controlled conditions.

Magnetic-Activated Cell Sorting

All antibody-based magnetic methods which can be used for cell sorting typically require the availability of antibody reagents to which biodegradable superparamagnetic beads of variable diameters (range: from tens of nanometers to hundreds of micrometers) are attached. The magnetic beads specifically bind to the cells of interest through antibody-mediated binding to cell surface epitopes. Cells that have such antibody-bound magnetic particles can be retained by passing a single cell suspension prepared from the sample through a magnet; by contrast, unlabeled cells would be removed. Afterward, the target cells can be collected separately from the other cells by turning off the magnetic field. For decades now, magnetic particles have been well established as standard tools for

the isolation of defined cell subsets in cell biology and immunology [18]. During this period, several different types of paramagnetic beads have been developed and made commercially available for cell sorting purposes. Among those most widely used are Dynabeads® (microbeads of 1–3 µm; Invitrogen, Carlsbad, Calif., USA) [59] and MACS® beads (nanobeads of 20–100 nm; Miltenyi Biotec, Bergisch Gladbach, Germany) [60].

Dynabeads are magnetisable superparamagnetic polystyrene spherical microbeads that are attached to target-specific antibodies which allow either isolation or depletion of intact cells directly from heterogeneous cell samples which contain distinct cell mixtures. Cell-containing samples that can be used include PB and other single-cell suspensions derived from body fluids, cell culture or disaggregated solid tissues [59]. One of the major advantages of these beads is that they do not require any special sample preparation step apart from their incubation with the sample (including the use of a separation column or centrifugation steps). In addition, Dynabeads also permit negative selection of the targeted cells when it is critical that no antibodies have been bound to any cell surface marker on the cell of interest, particularly when the sorted cells are to be used for further functional analyses [59]. However, this method also presents some limitations since detachment and removal of the beads after positive selection of the targeted cells is frequently required to perform downstream assays and analyses. In addition, their polyvalent binding can result in their endocytosis by labeled phagocytic cells and to an artificial activation of the labeled cells [61]. Furthermore, the binding capacity of antibody-conjugated Dynabeads to the cells of interest is slower than that of antibodies conjugated to smaller beads, e.g. nanobeads [54].

MACS technology is another widely used magnetic-based cell isolation procedure. This technique is based on the usage of a combination of high-gradient magnetic cell sorting and antibodies conjugated to biodegradable iron-based nanobeads. In the last decades, MACS has been extensively used for the isolation of both cells present in a sample at low frequencies (e.g. rare cells) and the HT purification of large cell numbers for both research and clinical applications [17]. Due to the small size of the paramagnetic particles bound to the antibody reagents, this technology requires usage of high-gradient magnetic cell separation columns filled with a matrix of ferromagnetic steel wool or iron spheres to retain the labeled cells in a magnetic field generated by a rather strong external magnet. When the magnetic field is turned off, the matrix can no longer retain the labeled cells, which can then be elut-

ed and purified [18]. Overall, positive selection is the fastest and most efficient way to isolate cells with high purity and yield. In addition, with this technology, however, negative cell selection after depletion of undesired cells, as well as sequential sorting strategies required when target cells cannot be specifically defined by a single marker, can also be efficiently performed depending on the specific cells or cell populations of interest and the availability of the appropriate combination of antibody reagents required for this purpose.

Flow Cytometry-Based Fluorescence-Activated Cell Sorting

Flow cytometry is a powerful technology that allows individualized analysis of both physical and chemical characteristics of single cells and other particles whenever these are (or have been) placed in suspension, as they pass one by one through one or more light sources (usually one or more lasers) [62]. The analyzed cells can be subsequently purified based on their unique light scatter and multiple fluorescence-associated (e.g. immunophenotypic) features. FACS is the process by which one or more different cell subsets and/or particles which have been analyzed can be physically isolated, simultaneously, from a heterogeneous cell sample. FACS sorting is typically characterized by high purity, even for cell populations which are represented at low frequencies (e.g. $<0.1\%$) in a sample [63]. Despite not being a bulk sorting method, FACS allows a relatively fast procedure for the isolation of multiple (e.g. ≥ 4) targeted cell populations simultaneously in a highly efficient way. Of note, FACS sorting can rely both on cell surface as well as intracellular proteins/markers. Cells purified by FACS technology can be directly placed into individual wells both for cell cloning [64] and polymerase chain reaction (PCR) analysis of single cells [65] or limiting dilutional cell culturing experiments.

For FACS-sorting methods, prior to their isolation, cells are labeled with a mixture of fluorescently conjugated antibodies that specifically bind surface and/or intracellular molecules on the cells of interest. Then, the labeled cells are delivered into a narrow and quickly flowing stream of liquid, so that cells pass aligned and one by one through a nozzle; this nozzle vibrates at a frequency of resonance to produce droplets containing individual cells at a defined distance from the nozzle. As the cells pass through the light source, a computer registers their individual light scatter and multiple fluorescent properties. Whenever a cell meets the pre-established criteria for a given cell population which is to be isolated, a mild elec-

trical charge is used to charge the drop where such a cell is going to be included. The charged droplets pass between two electrically charged metal plates, which deflect the charged droplets into different collection tubes (depending on the intensity and type of charge – positive versus negative charge – of the drop) or to the waste container (e.g. unwanted cells) [63].

Of note, sample preparation protocols and procedures performed prior to FACS analysis and sorting are also critically important for sorting efficiency. In fact, successful flow sorting highly depends on the state of the input sample. In this regard, samples should be as free as possible of cell/tissue debris, cell aggregates and/or erythrocytes since their presence can block the nozzle and thereby affect the sorting process, including contamination of the sorted cell fractions by unwanted cells and cell debris. In addition, cell concentration prior to sorting should also be adequate; very high cell concentrations will lead to an increased frequency of droplets containing more than one cell, resulting in both a lower purity and recovery of sorted cells, while very low cell concentrations can result in extremely long sorting times with decreased cell viability. Cell purity also depends on the level of distinction reached by the combination of fluorochrome-conjugated antibodies used among the distinct cell populations in the sample and the sample preparation protocol. Sample preparation under sterile conditions also contributes to improve cell viability.

Although FACS sorting is a highly efficient method for simultaneous purification of different (≥ 4) cell subsets with high purity and yield, this technology still remains a relatively low-speed approach, especially when large numbers of sorted cells ($>10^6$) are needed from a low-frequency ($<10^{-3}$) cell population. Moreover, the need for fluorochrome-conjugated monoclonal antibodies may alter cell viability and function; at the same time it is also associated with higher equipment and reagent costs [16].

Laser-Based Tissue Microdissection

A very important requirement of many research projects on cancer (and also other human diseases) is the availability of high-quality and appropriately selected tumoral and normal cells from affected tissues. For this purpose, LMD allows the isolation of pure cell populations from heterogeneous tissue specimens, including both frozen and formalin-fixed paraffin-embedded tissues, for a wide range of downstream assays, such as quantitative real-time PCR, cDNA microarrays, DNA sequencing, Western blot, 2-dimensional gel electrophoresis and

mass spectrometry [56]. The system is based on the combined use of an inverted light microscope equipped or not with a fluorescent module, and a laser device to facilitate visualization and 'selection' of the cells of interest; the laser device can be based either on an infrared laser-capture system or an ultraviolet laser-cutting system [56]. Since this approach allows for a good recovery of highly purified cells, it has been extensively used in a wide variety of applications in cell biology as well as pathology [66, 67] for multiple purposes including molecular characterization of cancer cells [68, 69], tissue chimerism [70], gene expression [71] and single-cell mutation analysis [72]. However, this cell sorting technique presents some disadvantages when applied alone in the routine daily work in biobanks since it only allows processing of small volumes of sample and the viability of the isolated cells, efficiency, yield and speed of this cell isolation method are low. In addition, another requirement of LMD is correct identification of the cells of interest in a complex tissue structure based on morphologic and/or immunophenotypic (e.g. single marker) characteristics by the operator, which typically requires supervision by a trained pathologist.

Other Cell Isolation Methods Based on Antibody Binding

In addition to flow cytometry-based and magnetic-associated cell isolation techniques, other antibody binding-based cell purification techniques have also been used in the past or are currently under development. For example, both low-density polypropylene floating beads coated with monoclonal antibodies [73] and protein-conjugated nickel particles used as heavy beads for gravitational sedimentation of the cells of interest [74] have been used for cell sorting purposes. In addition, specific cell populations can be depleted or enriched through complement-mediated lysis in combination with the usage of complement-binding monoclonal antibodies [75]. In addition, columns and polymers with antibody-immobilized surfaces have been developed to enrich for specific cell types in a sample (e.g. osteoblast cells as well as CD34⁺ umbilical cord blood cells) in an affinity/chromatography-based separation [76, 77]. Other antibody-based cell purification methods include magnetophoresis, defined as the movement of objects with respect to a surrounding medium caused by the net interaction of a magnetization with a magnetic field gradient [78], aptamer binding-based microfluidic devices for detection, enrichment and sorting of specific cell types [79], as well as other novel immunoaffinity-based methods for the isolation of cells in aqueous liquid-liquid biphasic systems, obtained either by the mixture of two

incompatible hydrophilic polymers or a polymer and an inorganic salt solution [80]. More recently, an immuno-magnetic-based column free cell isolation device for the purification of rare cell populations has been developed by BioCep Ltd. (Safed, Israel), that ensures purification of viable target cells suitable for further research and diagnostic laboratory analyses. However, cross-validation of these against other currently available cell purification techniques is still required.

Lab-on-a-Chip Cell Purification Methods

Apart from the cell separation approaches described above, there are several new lab-on-a-chip techniques for purification of specific cell populations which are currently under development and/or in an experimental phase of evaluation. Such lab-on-a-chip approaches operate in a microfluidic scale and are based on a label-free procedure for the isolation of the cells of interest. Among others, these include microscale filters or pillars that allow isolation of specific cell populations based on cell size and membrane deformability, as larger cells are prevented from navigating through, for example, the filter [46]. These lab-on-a-chip cell purification methods include (among other techniques): (1) field flow fractionation, which permits the isolation of cells along the length of a microfluidic channel by a combination of both the parabolic flow within the channel and an external field (e.g. an electric field or gravity) [81], (2) acoustophoresis, a membrane deformation or elasticity-based cell isolation method that separates cells when a high-pressure sound wave interacts with the cell [37], and (3) the dielectrophoresis method, which permits cell separation due to differential polarization of particles within a nonuniform electrical field [38].

Choosing Appropriate Cell Purification Methods

When selecting a cell isolation protocol and technology, there are four major features of the different procedures which are to be compared, which need to be considered upfront: cell purity (percentage of desired cells among the sorted fraction) reached with the individual approaches, cell recovery rate (absolute number of cells obtained after sorting compared to the number of target cells within the original sample prior to cell isolation), cell yield (the number of wanted cells recovered divided by the total number of wanted cells that were in the original sample prior to cell isolation, expressed as a percentage) and cell viability (undamaged, unmodified cells that can

be used with maximum efficiency for subsequent downstream applications they were purified for) [16, 19]. In turn, there are many other factors that also impact the choice of a specific cell isolation approach, such as the initial cell source, the features of the cell of interest, the required purity, recovery, yield and viability of the sorted cells, and the subsequent downstream applications for which the cells will be used, in addition to the need and potential availability of experienced personal and instrumentation. Finally, the overall cost of the process should also be considered (when it is an issue).

Overall, cell isolation procedures based on adhesion and filtration techniques are useful when large numbers of cells are needed regardless of their purity, without compromising cell viability for subsequent cell culture and functional assays [19, 82]. Similarly, density-gradient centrifugation is useful when dealing with samples that contain large cell numbers of cells to be isolated (e.g. PB) and cell purity is not a requirement. Alternatively, density-gradient centrifugation can also be used as a preparative step for sample enrichment in specific cell populations to be subsequently purified by other methods (e.g. FACS sorting) when an HT rate is required. However, antibody binding-based methods actually are the gold-standard techniques for the purification of specific subsets of cells from heterogeneous samples with high purity; these techniques are typically preferred when the downstream applications are not limited by the amount of biological material available, but they require highly pure and uncontaminated samples, such as quantitative real-time PCR approaches or gene expression analyses [82]. For antibody-mediated cell sorting techniques, detailed knowledge about the phenotype and function of the cell(s) type(s) of interest and the potential antibody-targeted molecules is critically important since some receptors are internalized after cell stimulation upon antibody binding, resulting in changes in the cell phenotype and the sorting efficiency [83]. In such cases, alternative negative selection procedures through which unwanted cells are depleted leaving the enriched cell type of interest untouched may be preferred. Moreover, detailed knowledge about the biology of the cell of interest and the pattern of expression of immunoglobulin receptors of the wanted cells as well as of the other cells present in the sample may also be critical to reduce the risk for nonspecific binding of antibodies, and thus the achievement of higher rates of purity [84].

Based on all the above, it may be said that antibody-binding-based sorting methods currently represent the best choice for the purification of specific cell subsets to be applied in different areas of biomedical research, particu-

larly those related to hematology, oncology and immune (or autoimmune) disorders. For example, paroxysmal nocturnal hemoglobinuria (PNH) is a hematological disease characterized by a deficient expression of glycosylphosphatidylinositol-anchored molecules on pathological cells affected by a mutation in the PIG-A gene. In this disease, analysis of the differential mRNA expression profile between normal and clonal (PNH) cells could be of high interest for the identification of potential molecular markers involved in determining the clinical consequences of, for example, hemolysis in the outcome of the disease. In order to obtain the maximum purity and yield of both the pathological and normal PB leukocyte subsets from PNH patients for subsequent molecular analyses, a combination of MACS- and FACS-sorting methods can be applied after a density-gradient centrifugation pre-enrichment step. This would allow fast purification of multiple normal and pathological PB cell subsets with the minimal cellular stress. Thus, from the isolated MNC, efficient purification of B cells can be achieved by MACS-positive selection followed by multiparameter FACS sorting of both PNH and normal B-cell populations. In turn, the negative MACS cell fraction can be used for FACS purification of PNH and normal subsets of monocytes and both CD4⁺ and CD8⁺ T cells. PNH and normal neutrophils can be directly sorted by FACS once erythrocytes are removed from the lower layer obtained after density-gradient centrifugation with an ammonium chloride-mediated red cell lysis procedure. Similarly, B-cell chronic lymphocytic leukemia is an interesting example in which MACS-sorting methods can be successfully applied for direct purification of B cells from these patients, in order to perform HT genetic analysis of DNA extracted from purified tumor versus nontumoral cells in the sample [85]. In turn, systemic mastocytosis could be an example of another disorder in which the application of FACS-sorting methods for direct purification of mast cells and other immune cells in a single step, could be of high interest for the correct diagnosis and prognostic stratification of the disease in individual patients through the evaluation of the presence of the KIT mutation in myeloid cells (neutrophils, monocytes), lymphoid cells (B lymphocytes), precursor cells (CD34⁺) and mesenchymal cells versus mast cells, as multilineage involvement by the KIT mutation has been associated with a high versus low rate of progression to more aggressive forms of the disease [27, 28].

For each of the three examples listed above, the overall costs of the process of purifying the different cell subsets of interest will highly depend on the overall number of cells to be purified from each cell subset. In turn, this will

also depend on the subsequent downstream applications to be performed with the isolated cells. In case of antibody-binding-based cell sorting methods, the overall costs of the purification processes are usually relatively high since the different reagents required are expensive (e.g. multiple fluorescent-conjugated monoclonal antibodies for multiparameter FACS sorting, as well as paramagnetic particles bound to antibody reagents together with high-gradient magnetic cell separation columns in the case of MACS methods). In order to minimize as much as possible the overall cell purification costs, different sample pretreatment steps may be applied, such as density-gradient centrifugation pre-enrichment steps as well as erythrocyte lysis procedures, which reduce the overall volume of reagents required for cell purification.

Once a specific cell isolation methodology has been chosen, adequate pretreatment of the sample prior to cell purification is also highly relevant since there are several important technological limitations that can seriously compromise the quality of the cell isolation process, and subsequently, the overall result. For example, the presence of cell clusters as a consequence of blood coagulation, incomplete tissue digestion, reestablishment of cell-cell contacts between dissociated cells after centrifugation or DNA release from dead cells [86–88] diminishes the efficiency of cell isolation procedures. Therefore, disrupting a tissue initially by mechanical methods and rotating the sample to improve enzymatic digestion [89, 90], together with filtering the sample to remove the vast majority of cell clusters, and the usage of magnesium and calcium-free buffers to prevent dissociated cells to reattach and form cell clusters [91], together with addition of DNase to digest the DNA released by dead cells, are steps which may all contribute to reduce cell clustering, and consequently to improve the cell purification efficiency of the process. Another technical issue to be considered once antibody-mediated cell purification techniques are used is the potential occurrence of nonspecific antibody binding to both dead cells and Fc receptors, which may also lead to reduced purity of the isolated cells [92]; nonspecific antibody binding can be reduced by both removing dead cells with a pre-enrichment step and/or by adding Fc-receptor-blocking reagents [93, 94].

HT Cell Sorting for Biobanks

Although the number of biobanks has grown exponentially all over the world in the last decade, storage of purified cell populations has not been included as a rou-

Table 2. Features to be met by HT cell sorting techniques and sample preparation procedures applied to biobanks

Features	
1	HT rate
2	Ability to process large volumes of sample
3	Simultaneous purification of multiple cell subsets
4	High efficiency
5	High cell recovery
6	High purity of isolated cells
7	Maintenance of cell viability
8	Work under cell stabilization conditions
9	Optimization of sorting protocols
10	Automation

tine procedure in the vast majority of the currently existing and active biobanks. A major reason for not incorporating cell purification methods in routine practice in most biobanks is probably due to the fact that most of the currently available techniques still have important limitations that make them not sufficiently efficient or useful to be applied routinely as HT cell purification approaches; usage of several methods in parallel is also frequently required. Therefore, both a clear need and a great opportunity exist for the development of preparative HT cell purification techniques for biobanks. The required HT cell sorting technology and sample preparation procedures would need to have the following features (table 2): (1) a HT rate in order to minimize cell purification time without compromising cell viability and/or altering RNA and protein expression profiles, (2) the ability to process large volumes of sample particularly when rare cell populations are to be purified, (3) simultaneous purification of multiple cell subsets coexisting in a sample, (4) high efficiency of both cell sorting and sample preparation processes, (5) high cell recovery to allow storage of enough materials from the sorted cells, particularly when paucicellular samples are treated (e.g. cerebrospinal fluid and vitreous fluid), (6) high purity (>95%) of the isolated cell populations, (7) the ability to maintain cell viability even after purification of cell subsets from solid tissues (i.e. tumor stem cells), (8) the ability to work under cell stabilization conditions, restricting or inhibiting activation of stress (e.g. hypoxia-induced) pathways, (9) optimization of sorting protocols as regards, for example, the reagents used to minimize the costs of the process, and (10) automation of the process as much as possible.

Currently, FACS and MACS technologies are the gold standard for single-cell sorting towards HT studies, al-

Table 3. Degree of purity of multiple populations of leukocytes purified from PB samples of healthy volunteers at BNADN

Sorting technology	Neutrophils	Monocytes	B cells	CD4 ⁺ T cells	CD8 ⁺ T cells
MACS	99.1±1.2 (86.8–100)	–	–	–	–
FACS	–	99.1±1.1 (87.1–100)	95.2±5.5 (70.1–99.9)	99.1±1.6 (76.9–100)	99.1±1.1 (92.9–100)

Results expressed as mean percentage of cells from the whole fraction of sorted/purified cells ± 1 SD (range).

though it is clear that efforts should be made to achieve higher throughput rates with these technologies (see above). More recently, droplet microfluidic platforms have provided a new way to perform single-cell and biomolecular studies for drug discovery, -omics studies and rare cell detection. Thus, a highly efficient microfluidic fluorescence-activated droplet sorter that combines the advantages of microtiter-plate screening and FACS technology has recently been developed [95]. However, although recent work in microfluidics has yielded methods for the encapsulation of cells in aqueous droplets and hydrogels, creating the potential for performing multiple single-cell experiments in parallel, commercial applications of HT microdroplet generation and downstream methods are still emerging for cells.

The Experience of the Spanish National DNA Bank Carlos III

The Spanish National DNA Bank Carlos III (BNADN) has become a reference biobank in Spain regarding the technical procedures developed and implemented for routine use (<http://www.bancoadn.org>). As a centralized scientific/technological platform whose main goal is to collect, process and provide researchers with biological human samples of high quality and data associated with such samples (given for research use by both healthy donors and patient volunteers), usage of cell purification procedures has become routine practice since 2011 for specific collections of samples. This is because of the increasing need for high-quality purified cell samples for the development of an increasing number of research projects, including the ICGC-CLL (International Cancer Genome Consortium on Chronic Lymphocytic Leukemia), which requires HT genetic analysis of DNA extracted from purified tumor versus nontumoral cells in the sample [85]. For this purpose, high-quality subproduct sample collections from different highly purified leukocyte subsets obtained from both healthy donors and pa-

tients with cancer and other clonal disorders, have been established by implementing a HT cell sorting protocol. Such protocol aims at obtaining a balanced maximum recovery, yield and purity of the sorted cell populations for further fractioning into subcellular components (e.g. DNA, RNA and miRNA) from each of the purified cell populations. For this purpose, a combination of the MACS and the FACS technologies has been developed and implemented for the purification of neutrophils and different subsets of MNC (e.g. monocytes, CD4⁺ T cells, CD8⁺ T cells and B cells) from PB samples. Briefly, MNC are first isolated after a density-gradient centrifugation pre-enrichment step, whereas erythrocytes are removed from the lower layer with an ammonium chloride-mediated red cell lysis procedure, in order to obtain a neutrophil-enriched cell fraction. In order to increase cell recovery, all tubes used to process the sample and collect cells are polypropylene tubes coated with 4% bovine serum albumin to prevent electrostatic charges on their surface prone to facilitate cell adherence. Afterward, efficient neutrophil purification is achieved by a MACS-based positive selection approach (mean cell recovery from the initial sample of 47.7 ± 14.1%). In parallel, PB monocytes, B cells, and both CD4⁺ and CD8⁺ T cells are efficiently purified by multiparameter FACS sorting with a mean overall purity of 99.1 ± 1.1, 95.2 ± 5.5, 99.1 ± 1.6 and 99.1 ± 1.1%, respectively (table 3). At present, BNADN has processed a total of 590 PB samples from healthy volunteers, from which 2,950 different cell populations have been obtained and stored in sufficient numbers in order to obtain enough DNA, RNA and miRNA from each purified cell population to be further used by the scientific community as control samples for the development of genomic and proteomic biomedical research projects for which approval by the BNADN External Ethical/Legal and Scientific Committees has been obtained in advance. Total DNA and RNA have been extracted from the purified cells with the Easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology Inc., Sangdaewon-dong, Korea), and stored at –80°C after controlling quality of the

nucleic acids which were extracted. These and other sub-cellular products are then made available for the scientific community as high-quality cell subproducts obtained from purified cell subsets.

Concluding Remarks

In recent years, a clear need has emerged for the availability of HT cell sorting methodologies to be implemented in routine biobanking activities for further use in biomedical research. Since currently available cell isolation methods still display important limitations that make them not cost-effective or useful enough to be applied routinely in biobanks as HT cell purification technologies, adaptation/translation of such procedures or development of new approaches for an HT biobanking format is required, given the importance of using high-quality

(purified) cell samples. Cell purification is particularly relevant to obtain reliable results when -omics technologies devoted to the analysis of gene, protein and metabolite expression profiles are to be used. Thus, biobanks should lead the field and make an effort to adapt, develop and implement cell purification technologies in their daily routine operational protocols whenever cell purification is foreseen as essential for obtaining reliable results.

Acknowledgements

This work was supported by grants from the Instituto de Salud Carlos III of the Ministry of Economy and Competitiveness of Spain: Plataforma de Recursos Biomoleculares y Bioinformáticos (PT13/0001/0037) and Plataforma de Biobancos (PT13/0010/0067). We thank M.L. Sánchez, P. Bárcena and O.M. Muñoz from the Cell Sorting Service of the University of Salamanca for their kind collaboration and technical support.

References

- 1 Biesecker LG, Spinner NB: A genomic view of mosaicism and human disease. *Nat Rev Genet* 2013;14:307–320.
- 2 Weischenfeldt J, Symmons O, Spitz F, Korbel JO: Phenotypic impact of genomic structural variation: insights from and for human disease. *Nat Rev Genet* 2013;14:125–138.
- 3 Gonzaga-Jauregui C, Lupski JR, Gibbs RA: Human genome sequencing in health and disease. *Annu Rev Med* 2012;63:35–61.
- 4 Apweiler R, Aslanidis C, Deufel T, Gerstner A, Hansen J, Hochstrasser D, Kellner R, Kubicek M, Lottspeich F, Maser E, Mewes HW, Meyer HE, Mullner S, Mutter W, Neumaier M, Nollau P, Nothwang HG, Ponten F, Radbruch A, Reinert K, Rothe G, Stockinger H, Tarnok A, Taussig MJ, Thiel A, Thiery J, Ueffing M, Valet G, Vandekerckhove J, Wagener C, Wagner O, Schmitz G: Approaching clinical proteomics: current state and future fields of application in cellular proteomics. *Cytometry A* 2009;75:816–832.
- 5 Perrin RJ, Fagan AM, Holtzman DM: Multimodal techniques for diagnosis and prognosis of Alzheimer's disease. *Nature* 2009;461:916–922.
- 6 Revill K, Tycko B: Epigenomic insights into common disease. *Genome Med* 2011;3:71.
- 7 Cortessis VK, Thomas DC, Levine AJ, Breton CV, Mack TM, Siegmund KD, Haile RW, Laird PW: Environmental epigenetics: prospects for studying epigenetic mediation of exposure-response relationships. *Hum Genet* 2012;131:1565–1589.
- 8 Merrick BA, London RE, Bushel PR, Grissom SF, Paules RS: Platforms for biomarker analysis using high-throughput approaches in genomics, transcriptomics, proteomics, metabolomics, and bioinformatics. *IARC Sci Publ* 2011;163:121–142.
- 9 Vlaanderen J, Moore LE, Smith MT, Lan Q, Zhang L, Skibola CF, Rothman N, Vermeulen R: Application of omics technologies in occupational and environmental health research: current status and projections. *Occup Environ Med* 2010;67:136–143.
- 10 Li SC, Martijn C, Cui T, Essaghir A, Luque RM, Demoulin JB, Castano JP, Oberg K, Giandomenico V: The somatostatin analogue octreotide inhibits growth of small intestine neuroendocrine tumour cells. *PLoS One* 2012;7:e48411.
- 11 Gupta R, Kim S, Taylor MW: Suppression of ribosomal protein synthesis and protein translation factors by Peg-interferon alpha/ribavirin in HCV patients blood mononuclear cells (PBMC). *J Transl Med* 2012;10:54.
- 12 Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R, Dohner H, Pollack JR: Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 2004;350:1605–1616.
- 13 Jacquemier J, Ginestier C, Rougemont J, Bardou VJ, Charafe-Jauffret E, Geneix J, Adelaide J, Koki A, Houvenaeghel G, Hassoun J, Marininchi D, Viens P, Birnbaum D, Bertucci F: Protein expression profiling identifies subclasses of breast cancer and predicts prognosis. *Cancer Res* 2005;65:767–779.
- 14 Sydor JR, Nock S: Protein expression profiling arrays: tools for the multiplexed high-throughput analysis of proteins. *Proteome Sci* 2003;1:3.
- 15 Szaniszló P, Wang N, Sinha M, Reece LM, Van Hook JW, Luxon BA, Leary JF: Getting the right cells to the array: gene expression microarray analysis of cell mixtures and sorted cells. *Cytometry A* 2004;59:191–202.
- 16 Orfao A, Ruiz-Argüelles A: General concepts about cell sorting techniques. *Clin Biochem* 1996;29:5–9.
- 17 Will B, Steidl U: Multi-parameter fluorescence-activated cell sorting and analysis of stem and progenitor cells in myeloid malignancies. *Best Pract Res Clin Haematol* 2010;23:391–401.
- 18 Grützkau A, Radbruch A: Small but mighty: how the MACS-technology based on nano-sized superparamagnetic particles has helped to analyze the immune system within the last 20 years. *Cytometry A* 2010;77:643–647.
- 19 Tomlinson MJ, Tomlinson S, Yang XB, Kirkham J: Cell separation: terminology and practical considerations. *J Tissue Eng* 2013;4:2041731412472690.
- 20 Riegman PH, Morente MM, Betsou F, de Blasio P, Geary P: Biobanking for better healthcare. *Mol Oncol* 2008;2:213–222.
- 21 Henderson GE, Cadigan RJ, Edwards TP, Conlon I, Nelson AG, Evans JP, Davis AM, Zimmer C, Weiner BJ: Characterizing biobank organizations in the U.S.: results from a national survey. *Genome Med* 2013;5:3.

- 22 Guo KT, SchAfer R, Paul A, Gerber A, Ziemer G, Wendel HP: A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high-specific DNA aptamers. *Stem Cells* 2006;24:2220–2231.
- 23 Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC: Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 2009;27:1006–1020.
- 24 Terry VH, Johnston IC, Spina CA: CD44 microbeads accelerate HIV-1 infection in T cells. *Virology* 2009;388:294–304.
- 25 Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, Sawyers CL, Weissman IL: Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* 2004;351:657–667.
- 26 Majeti R, Becker MW, Tian Q, Lee TL, Yan X, Liu R, Chiang JH, Hood L, Clarke MF, Weissman IL: Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proc Natl Acad Sci USA* 2009;106:3396–3401.
- 27 Teodosio C, Garcia-Montero AC, Jara-Acevedo M, Sanchez-Munoz L, Pedreira CE, Alvarez-Twose I, Matarraz S, Morgado JM, Barcena P, Matito A, Mayado A, Sanchez ML, Diez-Campelo M, Escribano L, Orfao A: Gene expression profile of highly purified bone marrow mast cells in systemic mastocytosis. *J Allergy Clin Immunol* 2013;131:1213–1224, 1224.e1–e4.
- 28 Escribano L, Alvarez-Twose I, Sanchez-Munoz L, Garcia-Montero A, Nunez R, Almeida J, Jara-Acevedo M, Teodosio C, Garcia-Cosio M, Bellas C, Orfao A: Prognosis in adult indolent systemic mastocytosis: a long-term study of the Spanish Network on Mastocytosis in a series of 145 patients. *J Allergy Clin Immunol* 2009;124:514–521.
- 29 Lopez-Corral L, Gutierrez NC, Vidriales MB, Mateos MV, Rasillo A, Garcia-Sanz R, Paiva B, San Miguel JF: The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. *Clin Cancer Res* 2011;17:1692–1700.
- 30 Schmidt-Hieber M, Gutierrez ML, Perez-Andres M, Paiva B, Rasillo A, Taberner MD, Sayagues JM, Lopez A, Barcena P, Sanchez ML, Gutierrez NC, San Miguel JF, Orfao A: Cytogenetic profiles in multiple myeloma and monoclonal gammopathy of undetermined significance: a study in highly purified aberrant plasma cells. *Haematologica* 2013;98:279–287.
- 31 Fernandez C, Santos-Silva MC, Lopez A, Matarraz S, Jara-Acevedo M, Ciudad J, Gutierrez ML, Sanchez ML, Salvador-Osuna C, Berrueto MJ, Diaz-Arias JA, Palomo-Hernandez AM, Colado E, Gonzalez N, Gallardo D, Asensio A, Garcia-Sanchez R, Saldana R, Cervero C, Carbone-Baneres A, Gutierrez O, Orfao A: Newly diagnosed adult AML and MPAL patients frequently show clonal residual hematopoiesis. *Leukemia* 2013;27:2149–2156.
- 32 Handgretinger R, Lang P, Schumm M, Taylor G, Neu S, Koscielna E, Niethammer D, Klingebiel T: Isolation and transplantation of autologous peripheral CD34+ progenitor cells highly purified by magnetic-activated cell sorting. *Bone Marrow Transplant* 1998;21:987–993.
- 33 To LB, Haylock DN, Simmons PJ, Juttner CA: The biology and clinical uses of blood stem cells. *Blood* 1997;89:2233–2258.
- 34 Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JJ, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH: Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;13:4279–4295.
- 35 Lanzoni G, Alviano F, Marchionni C, Bonsi L, Costa R, Foroni L, Roda G, Belluzzi A, Caponi A, Ricci F, Luigi Tazzari P, Pagliaro P, Rizzo R, Lanza F, Roberto Baricordi O, Pasquinelli G, Roda E, Paolo Bagnara G: Isolation of stem cell populations with trophic and immunoregulatory functions from human intestinal tissues: potential for cell therapy in inflammatory bowel disease. *Cytotherapy* 2009;11:1020–1031.
- 36 Mattanovich D, Borth N: Applications of cell sorting in biotechnology. *Microb Cell Fact* 2006;5:12.
- 37 Petersson F, Aberg L, Sward-Nilsson AM, Laurell T: Free flow acoustophoresis: microfluidic-based mode of particle and cell separation. *Anal Chem* 2007;79:5117–5123.
- 38 Hu X, Bessette PH, Qian J, Meinhardt CD, Daugherty PS, Soh HT: Marker-specific sorting of rare cells using dielectrophoresis. *Proc Natl Acad Sci USA* 2005;102:15757–15761.
- 39 Nagase K, Mukae N, Kikuchi A, Okano T: Thermally modulated retention of lymphocytes on polymer-brush-grafted glass beads. *Macromol Biosci* 2012;12:333–340.
- 40 Didar TF, Tabrizian M: Adhesion based detection, sorting and enrichment of cells in microfluidic lab-on-chip devices. *Lab Chip* 2010;10:3043–3053.
- 41 Miller RG, Phillips RA: Separation of cells by velocity sedimentation. *J Cell Physiol* 1969;73:191–201.
- 42 Poynton CH, Dicke KA, Culbert S, Frankel LS, Jagannath S, Reading CL: Immunomagnetic removal of calla positive cells from human bone marrow. *Lancet* 1983;1:524.
- 43 Strelkauskas AJ, Teodorescu M, Dray S: Enumeration and isolation of human T and B lymphocytes by rosette formation with antibody-coated erythrocytes. *Clin Exp Immunol* 1975;22:62–71.
- 44 Autebert J, Coudert B, Bidard FC, Pierga JY, Descroix S, Malaquin L, Viovy JL: Microfluidic: an innovative tool for efficient cell sorting. *Methods* 2012;57:297–307.
- 45 Hosokawa M, Hayata T, Fukuda Y, Arakaki A, Yoshino T, Tanaka T, Matsunaga T: Size-selective microcavity array for rapid and efficient detection of circulating tumor cells. *Anal Chem* 2010;82:6629–6635.
- 46 Ji HM, Samper V, Chen Y, Heng CK, Lim TM, Yobas L: Silicon-based microfilters for whole blood cell separation. *Biomed Microdevices* 2008;10:251–257.
- 47 Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, Fleisher M, Stadler W, Datar RH, Tai YC, Cote RJ: Portable filter-based microdevice for detection and characterization of circulating tumor cells. *Clin Cancer Res* 2010;16:5011–5018.
- 48 Bauer KD, Keng PC, Sutherland RM: Isolation of quiescent cells from multicellular tumor spheroids using centrifugal elutriation. *Cancer Res* 1982;42:72–78.
- 49 Chavez-Crocker P, Garrido N, Ahearn GA: Copper transport by lobster hepatopancreatic epithelial cells separated by centrifugal elutriation: measurements with the fluorescent dye Phen Green. *J Exp Biol* 2001;204:1433–1444.
- 50 Worthington RE, Nakeff A: Thromboxane synthesis in megakaryocytes isolated by centrifugal elutriation. *Blood* 1981;58:175–178.
- 51 Schwarze PE, Pettersen EO, Tolleshaug H, Seglen PO: Isolation of carcinogen-induced diploid rat hepatocytes by centrifugal elutriation. *Cancer Res* 1986;46:4732–4737.
- 52 Lindahl PE: Principle of a counter-streaming centrifuge for the separation of particles of different sizes. *Nature* 1948;161:648.
- 53 Bonner WA, Hulett HR, Sweet RG, Herzenberg LA: Fluorescence activated cell sorting. *Rev Sci Instrum* 1972;43:404–409.
- 54 Miltenyi S, Muller W, Weichel W, Radbruch A: High gradient magnetic cell separation with MACS. *Cytometry* 1990;11:231–238.
- 55 Carroll S, Al-Rubeai M: ACSD labelling and magnetic cell separation: a rapid method of separating antibody secreting cells from non-secreting cells. *J Immunol Methods* 2005;296:171–178.
- 56 Liu A: Laser capture microdissection in the tissue biorepository. *J Biomol Tech* 2010;21:120–125.
- 57 Beaujean F: Methods of CD34+ cell separation: comparative analysis. *Transfus Sci* 1997;18:251–261.
- 58 Wysocki LJ, Sato VL: 'Panning' for lymphocytes: a method for cell selection. *Proc Natl Acad Sci USA* 1978;75:2844–2848.
- 59 Neurauter AA, Bonyhadi M, Lien E, Nokleby L, Ruud E, Camacho S, Aarvak T: Cell isolation and expansion using Dynabeads. *Adv Biochem Eng Biotechnol* 2007;106:41–73.
- 60 Abts H, Emmerich M, Miltenyi S, Radbruch A, Tesch H: CD20 positive human B lymphocytes separated with the magnetic cell sorter (MACS) can be induced to proliferation and antibody secretion in vitro. *J Immunol Methods* 1989;125:19–28.
- 61 Oren A, Husebo C, Iversen AC, Austgulen R: A comparative study of immunomagnetic methods used for separation of human natural killer cells from peripheral blood. *J Immunol Methods* 2005;303:1–10.

- 62 Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M: The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem* 2002;48:1819–1827.
- 63 Davies D: Cell separations by flow cytometry. *Methods Mol Med* 2001;58:3–15.
- 64 Battye FL, Light A, Tarlinton DM: Single cell sorting and cloning. *J Immunol Methods* 2000;243:25–32.
- 65 Williams C, Davies D, Williamson R: Segregation of delta F508 and normal CFTR alleles in human sperm. *Hum Mol Genet* 1993;2:445–448.
- 66 Agar NS, Halliday GM, Barnetson RS, Jones AM: A novel technique for the examination of skin biopsies by laser capture microdissection. *J Cutan Pathol* 2003;30:265–270.
- 67 Okuducu AF, Hahne JC, Von Deimling A, Wernert N: Laser-assisted microdissection, techniques and applications in pathology (review). *Int J Mol Med* 2005;15:763–769.
- 68 Huang C, Yang L, Li Z, Yang J, Zhao J, Dehui X, Liu L, Wang Q, Song T: Detection of CCND1 amplification using laser capture microdissection coupled with real-time polymerase chain reaction in human esophageal squamous cell carcinoma. *Cancer Genet Cytogenet* 2007;175:19–25.
- 69 Jensen LH, Cruger DG, Lindebjerg J, Byriel L, Bruun-Petersen G, Jakobsen A: Laser microdissection and microsatellite analysis of colorectal adenocarcinomas. *Anticancer Res* 2006;26:2069–2074.
- 70 Kleeberger W, Rothamel T, Glockner S, Lehmann U, Kreipe H: Laser-assisted microdissection and short tandem repeat PCR for the investigation of graft chimerism after solid organ transplantation. *Pathobiology* 2000;68:196–201.
- 71 Esposito G: Complementary techniques: laser capture microdissection – increasing specificity of gene expression profiling of cancer specimens. *Adv Exp Med Biol* 2007;593:54–65.
- 72 Nakamura N, Ruebel K, Jin L, Qian X, Zhang H, Lloyd RV: Laser capture microdissection for analysis of single cells. *Methods Mol Med* 2007;132:11–18.
- 73 Hirn-Scavennec J, Brailly H, Maraninchi D, Mannoni P, Mawas C, Delaage M: Elimination of leukemia cells from human bone marrow using floating beads. *Transplantation* 1988;46:558–563.
- 74 Recktenwald D, Radbruch A: *Cell Separation Methods and Applications*. New York, M. Dekker, 1998.
- 75 Sharp TG, Sachs DH, Fauci AS, Messerschmidt GL, Rosenberg SA: T cell depletion of human bone marrow using monoclonal antibody and complement-mediated lysis. *Transplantation* 1983;35:112–120.
- 76 Mahara A, Yamaoka T: Continuous separation of cells of high osteoblastic differentiation potential from mesenchymal stem cells on an antibody-immobilized column. *Biomaterials* 2010;31:4231–4237.
- 77 Kumar A, Srivastava A: Cell separation using cryogel-based affinity chromatography. *Nat Protoc* 2010;5:1737–1747.
- 78 Schneider T, Karl S, Moore LR, Chalmers JJ, Williams PS, Zborowski M: Sequential CD34 cell fractionation by magnetophoresis in a magnetic dipole flow sorter. *Analyst* 2010;135:62–70.
- 79 Xu Y, Phillips JA, Yan J, Li Q, Fan ZH, Tan W: Aptamer-based microfluidic device for enrichment, sorting, and detection of multiple cancer cells. *Anal Chem* 2009;81:7436–7442.
- 80 Sousa AF, Andrade PZ, Pirzgalska RM, Galhaz TM, Azevedo AM, da Silva CL, Aires-Barros MR, Cabral JM: A novel method for human hematopoietic stem/progenitor cell isolation from umbilical cord blood based on immunoaffinity aqueous two-phase partitioning. *Biotechnol Lett* 2011;33:2373–2377.
- 81 Vykoukal J, Vykoukal DM, Freyberg S, Alt EU, Gascoyne PR: Enrichment of putative stem cells from adipose tissue using dielectrophoretic field-flow fractionation. *Lab Chip* 2008;8:1386–1393.
- 82 Ferrero H, Delgado-Rosas F, Garcia-Pascual CM, Monterde M, Zimmermann RC, Simon C, Pellicer A, Gomez R: Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study. *Hum Reprod* 2012;27:1781–1789.
- 83 Chatenoud L, Bluestone JA: CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* 2007;7:622–632.
- 84 McCloskey KE, Moore LR, Hoyos M, Rodriguez A, Chalmers JJ, Zborowski M: Magnetophoretic cell sorting is a function of antibody binding capacity. *Biotechnol Prog* 2003;19:899–907.
- 85 Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, et al: Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011;475:101–105.
- 86 Renner WA, Jordan M, Eppenberger HM, Leist C: Cell-cell adhesion and aggregation: influence on the growth behavior of CHO cells. *Biotechnol Bioeng* 1993;41:188–193.
- 87 Pisetsky DS, Fairhurst AM: The origin of extracellular DNA during the clearance of dead and dying cells. *Autoimmunity* 2007;40:281–284.
- 88 Steinberg MS: 'ECM': its nature, origin and function in cell aggregation. *Exp Cell Res* 1963;30:257–279.
- 89 Klein AB, Witonsky SG, Ahmed SA, Holladay SD, Gogal RM Jr, Link L, Reilly CM: Impact of different cell isolation techniques on lymphocyte viability and function. *J Immunol* 2006;27:61–76.
- 90 Noel JS, Zucker RM, Wu NC, Demaray SY: The dissociation of transplantable tumors. *J Histochem Cytochem* 1977;25:544–553.
- 91 Booth C, O'Shea JA, Potten CS: Maintenance of functional stem cells in isolated and cultured adult intestinal epithelium. *Exp Cell Res* 1999;249:359–366.
- 92 Stewart CC, Stewart SJ: Multiparameter analysis of leukocytes by flow cytometry. *Methods Cell Biol* 1994;41:61–79.
- 93 Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV: Dead cell discrimination with 7-amino-actinomycin d in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* 1992;13:204–208.
- 94 Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M: Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J Immunol Methods* 2006;313:199–208.
- 95 Baret JC, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, Rick C, Samuels ML, Hutchison JB, Agresti JJ, Link DR, Weitz DA, Griffiths AD: Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip* 2009;9:1850–1858.