Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

CHIMICA

Ciclo XXIX

Settore Concorsuale di afferenza: CHIM/01

Settore Scientifico disciplinare: 03/A1

DEVICE FOR SEPARATION OF STEM CELLS FROM ADULT TISSUES, SUITABLE FOR REGENERATIVE MEDICINE RESARCH: FROM THE IDEA TO THE READY-TO-MARKET STAGE

Presentata da: **KRISTEL MARTINELLI**

Coordinatore Dottorato **Relatore**

Prof. Aldo Roda **Prof. Pierluigi Reschiglian**

Esame finale anno 2017

Dott. Kristel Martinelli
Supervisore: Prof. Pierluigi Reschiglian
Curriculum: Scienze Chimiche
Indirizzo: Chimica Analitica
Titolo della Tesi: Device for separation of stem cells from adult tissues, suitable for regenerative medicine research - from the idea to the ready-to-market stage

During the three years of her PhD, Kristel Martinelli focused her research project on design, instrumental development and application of a novel, flow-assisted technology for the separation and selection of cells, in particular stem cells. The importance of this technology concerns the particularly gentle method by which cells are swept down the separation device and are, then separated. This allows full maintenance of the physiological characteristic of the cells, a key point to make cells, and more specifically stem cells, able to be used for further biological characterization or cell culture. The novel technology implements a cluster of patents of the University of Bologna (IT1371772, US 8263359, CA2649234) into an instrumentation that will be addressed to the market. The physical principle underpinning this novel technology is that Earth's gravity assists the dynamic fractionation of cells suspended in a liquid stream based on differences in physical-morphological properties (size, shape, density, surface features) of the cells. This turns out into a unique tool for non-invasive cell sorting. "Non-invasive" means that cells can be separated at a highly pure level (>90%) just by physical means. This avoids the use of surface immune-markers that can modify cell biology and promote the unpredicted outcome of their molecular characterization.

The candidate focused on the analytical, instrumental aspects of the technology using, first, cell samples, either from cell cultures or from real, raw samples. Using cells of different nature, particular emphasis was given to the application to mesenchymal stem cells and on the relevant search of biocompatibility and

adequacy of the technical solutions chosen for developing the instrumental prototypes into a possible, future product.

Indeed the candidate acquired good experience also in validation and development of flow-assisted separation methods, analytical instrumentation design and development, techniques for cell characterization like flow cytometry and related FACS, magnetic-assisted cell sorting (MACS), immunofluorescence, microscopy, cell culturing and cloning. The possible orientation to a market outcome of the PhD project made the candidate getting acquainted also on strategic marketing, communication techniques and business planning.

During the PhD project, the candidate attended national and international congresses and events, also presenting poster and oral communications, among which, in 2016, a presentation to MEDTEC Europe, Stuttgart (Germany), to the “International Summer School - Innovation and Technology Management in Medical and Pharmaceutical Biotechnology”, of the Bologna Business School, and to the “Y-RICH-Young Research Ideas”, Università di Roma “La Sapienza”. With an entrepreneurship project based on the technology developed during her PhD, the candidate got the final of the “Premio Marzotto 2014”, the Italian, most important competition for startup projects.

The candidate has developed understanding of all the issues involved. She acquired a good mastery of the experimental techniques, demonstrated skills of organization, coordinated well with laboratory colleagues, and showed ability to relate with external collaborators. She also developed “soft skills” that will make her able to be competitive on business-related activities.

In my opinion Kristel Martinelli has carried out a very good work for the thesis.

The Board expresses a very good score on the activity carried out by the candidate during the whole cycle of doctorate and considers her worthy to attain the PhD in Chemistry.

TABLE OF CONTENTS

Aim of the study and Introduction

My PhD research project was focused on the development of a brand new instrumentation for the separation of particles, chiefly of interest for medical and clinical issues, thanks to the advantage related to the preservation of the sample or “minimum manipulation” in the medical field. The competitive advantage, respect to the state of art of current cell separation techniques, is the unique and specific separation method based solely on the morpho-physical proprieties of particles moving inside the flow of a capillary separation channel. The combination of forces developed inside the channel and due to the flow composed of a biocompatible liquid and the Earth Gravitation Field, joined to the geometries of the system and the sample manipulation procedures, allows the separation in time and in space of particles with different physical proprieties. The different population can be collected, characterized and reused for further applications. This feature is particularly relevant when the preservation of the native proprieties of the sample is unavoidable and when the traditional parameters of traditional techniques are not effective or not efficient for the separation of the species contained in a suspension. This is a significant limit when we’re talking about drugs or therapies presenting peculiar characteristics, as the stromal cell population and in particular the mesenchymal stem cells (MSCs), where the complexity of the sample in reason of their “not specialized” state, leads to a really heterogeneous population, preventing the identification by traditional protocols. This heterogeneity can be tapped by the novel instrumentation, first of all in the regenerative medicine field that uses cell based therapy, most of them belonging from stem cell or derived or progenitors cells, samples not to be compromised during the analysis or the separation procedures. MSCs are the most promising stem cell type for cell-based therapies since they are virtually present in all adult tissues and possess tissue regenerative and immunosuppressive properties. MSCs are adult stem cells which can be induced to enter various mesenchymal lineage pathways to differentiate towards the more specialized osteogenic, chondrogenic, myogenic and adipogenic cell lineages. They appear to be particularly suitable for clinical

applications in the fields of cell therapy and tissue reconstruction, for treatments of compromises organs and tissues. Mesenchymal cells are located in all human tissues, but some tissues are particularly rich in MSCs such as the fatty tissue, spinal cord (bone marrow), dental pulp and neonatal tissues. Starting from these sources I studied the behavior of stem cells before, during and after the separation procedure to build up the technology respect the biological requirements of manipulation and optimizing the methods respect the proprieties of stemness of the different fractions resulting from the separation.

Moving from this request, I developed a technology that builds on the patented method (IT1371772, US 8263359, CA2649234) for gentle stem cell separation and evolves into an instrumentation serviceable and scalable to be brought on the market and available for “stringent criteria of manipulation” applications.

To meet the demands of the market, I considered the whole project in order to insure an organic and coordinated development of the product with the aim to guarantee a fully functional product, thought to be appropriate for the beta-testing and the first placement on the market. Briefly, the project of my research was to transform a method of separation compliant for cell samples into a full automated product usable by not specialized personnel, related with a full protocol of separation portfolio which include a panel of characterization off-&-on-line cell population and subpopulation, ensuring the compliance of the whole process with Class IIb Medical Device certification. This latter aspect worked as the “shadow guideline” moving with the project progress, starting from the suppliers, materials and manufacturing techniques/ procedures, transports, destination of use and environment, and finishing with the biologic cell sample selection and timing, cell characterizations, sterilization and operational methods dependent on methodical daily working rules of the target client. Coordinating all these aspects allowed to gain a first instrumentation boasting the principal features for future medical applications or immediate “low scale” amount of cells in particular therapies.

The resulting product developed in compliance with engineering and biotechnological requirements, merged with industrial and production and

strategies, in order to helpfully supply the Regenerative Medicine sector, is called Celector®.

Chapter 1

Stem cells and Regenerative Medicine

Stem Cells: Basics

Stem cells are a population of precursor cells that are capable of developing into many different cell types in the body. When a stem cell divides, each new cell has the potential either to remain a stem cell or differentiate into another type of cell with a more specialized function. Stem cells are distinguished from other cell types in the body by capability of self- renewal and under certain conditions induced to differentiate into specific cells. In some organs, (for example the bone marrow, or skin), stem cells regularly divide to repair and replace worn out tissues which was discovered in the early 1960s, and knowledge about their characteristics and composition has come a long way. The existence of stem cells was first demonstrated in 1960 by Till and McCulloch in a study on hematopoiesis. The establishment of the concept of hematopoietic stem cells (HSCs) was followed by the discovery of tissue stem cells in other organs in mammals, for example,

epithelial stem cells, neural stem cells, and intestinal stem cells[1]. Stem cells are important for living organisms for many reasons. In the 3 to 5 day old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lung, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease

Stem cells differ from other kinds of cells in the body. All stem cells regardless of their source have three general properties: Stem cells are unspecialized. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures and cannot work with its neighbors to pump blood through the body (like a heart muscle cell); it cannot carry molecules of oxygen through the bloodstream (like a red blood cell); and it cannot fire electrochemical signals to other cells that allow the body to move or speak (like a nerve cell).

However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells. Stem cells are capable of dividing and renewing themselves for long periods. When cells replicate themselves many times over it is called proliferation. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal. Stem cells can give rise to specialized cells. When unspecialized stem cells give rise to specialized cells, the process is called differentiation. Scientists are just beginning to understand the signals inside and outside cells that trigger stem cell differentiation. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA,

[1] Fuchs and Segre, 2000

and carry coded instructions for all the structures and functions of a cell. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells' predicted lineage (that is, brain stem cells that differentiate into blood cells or blood forming cells that differentiate into cardiac muscle cells and so forth). This reported phenomenon is called transdifferentiation.

Types of stem cells

Stem cells can be divided based on their self-renewal and potency[2]. Self-renewal is the ability to go through numerous cycles of cell division while maintaining the undifferentiated state while potency is the capacity to differentiate into specialized cell types. Based on the potency, stem cells can be divided into five groups. The first type is the totipotent stem cells. These cells can differentiate into embryonic and extraembryonic cell types. These cells are produced by fusion of an egg and sperm cell. The second type is pluripotent stem cells. These cells are the progenies of totipotent cells and can differentiate into almost all cells except extraembryonic cell types. The cell has the potential to differentiate to any of the three germ layers are examples of this type**.** The third type is the multipotent stem cells which can differentiate into a number of cells, but only those of a closely related family of cells. The fourth type is the oligopotent stem cells. These cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells. Finally, the fifth group is the unipotent cells. Therefore, all types of stem cells have the ability of self-renewal but their potency is different and depends on the source that

[2] Zhang and Cheng, 2013

they have arisen from[3]. Based on their source, stem cell can also be classified as embryonic, fetal, adult, amniotic cord blood and Induced pluripotent.

Stem cell bioprocessing[4]

The success of stem cell bioprocessing relies on robust and reproducible culture conditions and processes. For stem cell bioprocessing, this includes the scale-up of stem cells to a differentiated end product of sufficient quality and quantity for clinical and commercial goals. Automation and the use of an efficient bioprocess paradigm are imperative for the creation of successful clinical products. The design principles [5]pertinent to stem cell bioprocessing can be categorized into three groups: process components; process requirements and process function, as summarized in Figure 1. A combination of generic, 'off-the-shelf' and personalized manufacturing paradigms must be considered as no single technology satisfies all requirements[6] (Figure 1.1)

[3] Yao et al., 2012
[4] Dubie et al. **Journal of Cell Biology and Genetics,** Vol. 4(4), pp. 40-52,,2014
[5] Lim et al., 2007
[6] Mark et al., 2009

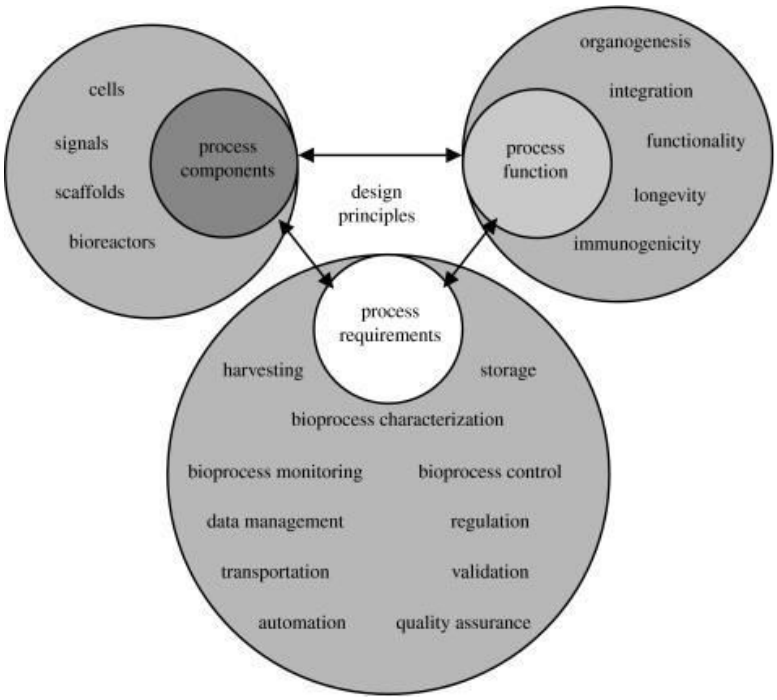


Figure 1.1 Design principles for stem cell bioprocesses. Source: (Lim et al., 2007).

MSC in Regenerative Therapy

The regenerative potential of MSC isolated from different tissues has been shown to undergo alteration according to the tissue of isolation[7][8]. It has been shown that BM-MSC possess a higher potential in giving rise to osteoblasts and chondrocytes[9], whereas adipose tissue-derived MSC (AT-MSC) have been shown to contribute more successfully to capillary-like network formation in vitro as well as vasculogenesis in vivo [85, 86]. Umbilical cord blood- (UCB-) MSC also showed a high potency in giving rise to pericytes during vasculogenesis, whereas their potential for osteogenic differentiation has been shown to diminish compared to

[7] A. Reinisch, N. A. Hofmann, A. C. Obenauf et al., *Blood*, vol. 113, no. 26, pp. 6716–6725, 2009

[8] N. A. Hofmann, A. Ortner, R. O. Jacamo et al., *PLoS ONE*, vol. 7, no. 9, Article ID e44468, 2012.

[9] *International Journal of Molecular Sciences*, vol. 14, no. 9, pp.17986–18001, 2013

BM-MSC [10], which still play as the gold standard for osteogenic differentiation and regeneration. AMN-MSC were also shown to successfully participate in neurogenesis, whereas such a regenerative potential has not been distinguished in UC-MSC[11]. Amniotic membrane-derived MSC, however, have not been shown to participate in the process of vasculogenesis as successfully as UC-, UCB-, AT-, and BM-MSC did [86]. Despite the fact that DPSC and BM-MSC are regulated by similar factors and they also possess a similar protein expression profile, these populations have been shown to alter significantly in their proliferative capacity in vitro and, more importantly, in their regenerative capacity in vivo[12]. BM-MSC give rise to bone tissue in the mouse model under treatment as described in studies. The chondrogenic and adipogenic potential of BM-MSC has been higher compared to that of DPSC[13]. Conversely, the neurogenic differentiation potential of dental mesenchymal stem cells might be more robust compared to that of BMMSC, since these cells possess neural crest origin. BM-, dental pulp- (DP-), and adipose tissue- (AT-) derivedMSC have revealed a greater promise in regenerative therapy since these adult stem cells might promote patientspecific regenerative interventions. MSC are attractive alternatives for regeneration of the injured and/or deficient cells and tissues due to their multipotent differentiation capacity as well as their immunomodulatory and anti-inflammatory properties through cellular crosstalk and production of bioactive molecules. MSC have the unique potential either to directly participate in regeneration and repair processes or to play an immune

[10] A. Ardeshirylajimi, M. Mossahebi-Mohammadi, S. Vakilian et al., *Cell Proliferation*, vol. 48, no.1, pp. 47–58, 2015.

[11] E. Y. Kim, K.-B. Lee, and M. K. Kim, *BMB Reports*, vol. 47, no. 3, pp. 135–140, 2014.

[12] S. Shi, P. G. Robey, and S. Gronthos,, *Bone*, vol. 29, no. 6, pp. 532–539, 2001.
[13] W. Zhang, X. F.Walboomers, S. Shi, M. Fan, and J. A. Jansen, *Tissue Engineering*, vol. 12, no. 10, pp. 2813–2823, 2006.

modulatory role to enhance treatment of autoimmune diseases such as type 1 diabetes (T1D).

Focus on the most interesting source of mesenchymal stem cells: adipose tissue derived MSCs

In the last decade, rapid evolution in the biology and biotechnology's fields led to development of different viable cell-based medical applications, which hold a high potential in treatment of several diseases still lacking a specific therapy. In this context, stem cells are the most promising source of cells, mainly because of their limitless avalaibility and easy manipulation (Guilak et al, 2010).

Stem cells can be defined as cells with the capability of generating daughter cells (self-renewal property) and having multi-lineage differentiation capacity (EMA/CAT/571134/2009). Stem cells are able to proliferate in an undifferentiated form and include:

- embryonic stem cells derived from blastocysts (hESC);
- adult and/or somatic stem cell, including:
- haematopoietic stem cells (HSCs);
- mesenchymal stromal/stem cells (MSCs);
- tissue-specific progenitor cells, unipotent cells that can develop into a limited panel of tissues;
- induced pluripotent stem cells (iPSs).

Among all these types of stem cells, MSCs are the most promising for cell-based therapies since they are virtually present in all adult tissues ([14]) and possess tissue regenerative (Pittenger et al., 1999) and immunosuppressive properties (Aggarwal et al., 2005).

MSCs are adult stem cells which can be induced to enter various mesenchymal lineage pathways to differentiate towards the more specialized osteogenic, chondrogenic, myogenic and adipogenic cell lineages. Although bone marrow has been considered for years the classical reservoir of MSCs (BM-MSCs), several new sources are currently under investigation. In particular, the adipose tissue has been proven to be an increasingly attractive source of MSCs for mesenchymal tissues regeneration[15], since fat is easily obtainable in large quantities and it yields a cells number per gram of tissue which is 500-fold higher than the bone marrow.[16]

MSCs isolated from different tissues differently reacts to inductive molecules, thus reflecting the characteristics of tissues of origin (Caplan, 2008); however, in culture, BM-MSCs and adipose-derived MSCs (ASCs) share an important combination of features:

1) adherence to plastic [17][18]

2) morphology[19];

[14] Caplan, 2010; da Silva Meirelles et al., 2006
[15] Locke et al., 2009
[16] Fraser et al., 2006

[18] Dominici et al., 2006; Zuk et al., 2002

3) immunophenotype[20];

4) differentiation capacity;

5) immunosuppressive capacity[21].

Therefore, also considering the same embryonic mesodermic origin, it is likely to account ASCs as a peripheral MSCs lineage, supporting their use in several therapeutic applications. In particular, ASCs hold high potentials in orthopaedic tissue-engineering field, since they both promote osteogenesis at break sites and increase bone grafts integration[22]. Moreover, ASCs were shown to possess immunosuppressive and anti-rejection capacities; this finding rationally supports their allogenic use.

MECHANISMS OF ACTION

The therapeutic value of MSCs is based on a number of intrinsic characteristics, briefly listed and discussed below, which are shared by both BM-MSCs and ASCs:

1) differentiation ability;

2) trophic activity;

3) immunomodulatory capacity

---

[19] Zuk et al., 2002
[20] Peroni et al, 2008
[21] Puissant et al., 2005;
McIntosh et al., 2006
[22] Tapp et al., 2008

1) Differentiation ability

MSCs have been originally isolated and characterized to study their ability to differentiate into a broad spectrum of mesenchymal tissues, such as bone, cartilage, tendon, fat, muscle and marrow stroma. Firsts therapeutic applications were thus proposed, basing on the mere tissue engeneering logic that lineage-oriented stem cells could reconstruct a specific site of application[23]. However, several pre-clinical studies demonstrated that MSCs-induced functional recovery of treated injured tissues occurs without a substantial differentiation of injected MSCs towards tissue-related phenotypes. Therefore, others mechanisms of action must be involved and differentiation should be considered as a secondary feature.

New insights in MSCs pharmacodynamic depict this multipotent cell lineage as intelligent, injury-site specific, multidrug release system (Caplan, 2010). In fact, MSCs could be recluted by injured organs and, while chemoattracted by the proinflammatory cytokine tumor necrosis factor-α (TNF-α)[24], home to sites of inflammation where they secrete a massive amount of bioactive agents, both trophic and immunomodulatory .

2) Trophic activity

It is considered “trophic activity” the MSCs ability to stimulate host regeneration trhough paracrine secretion of a serie of molecules that induce the following physiological responses:

[23] Wagner et al., 2009
[24] Ponte et al., 2007

a) inhibition of apoptosis with consequent limitation of the damaged field;

b) inhibition of scarring and fibrosis in the site of injury, thus reducing severe post-lesions fibrogenesis;

c) stimulation of angiogenesis;

d) stimulation of proliferation of tissue-specific regenerative progenitors.

Trophic activity of MSCs represent a key feature in bone regeneration and graft survival. In fact, angiogenesis and consequent avalaibility of blood supply are crucial, both for reformation of new structural osseous tissue and for success of engineered scaffolds engraftment.

In addition, MSCs-induced stimulation of tissue progenitors to divide and differentiate into functional regenerative units, represents one of the most important properties underlying organs regeneration.

3) Immunomodulatory capacity

MSCs are known to avoid allogeneic rejection (Ryan et al., 2005); powerful immunomodulatory and antinflammatory properties of this cell lineage are the most important pharmacological rationals justifying their allogeneic uses. Three broad mechanisms contribute to MSCs anti-rejection ability:

a) MSCs are hypoimmunogenic themselves; even if there are still some controversial results about MSCs cell surface expression of major histocompatibility complexes (MHC), many researches suggest that these cells are

MHC-II negative (McIntosh et al., 2009; Ryan et al., 2005). Absence of MHC-II gives to MSCs the useful potential to escape host CD4+ T cells recognition;

b) MSCs are able to suppress proliferation and cytokine secretion of natural killer (NK) cells by cell-to-cell direct interaction (Sotiropoulou et al., 2006);

c) MSCs extensively secrete a wide range of bioactive molecules, which create a surrounding immuno-suppressive milieu. Prostaglandin E2 (PGE-2) was found to be a central effector of several MSCs-mediated effects on immune system; in fact, it has been shown that MSCs-secreted PGE-2 has powerful inhibiting activities on dendritic-1 (DC-1), T and NK cells proliferation and secretory profile (Aggarwal and Pittenger, 2005; Sotiropoulou et al., 2006). In the meantime, PGE-2 also increases DC-2 cells secretion of interleukin-10 (IL-10), which, in turn, suppresses the outcome of TNF-α and interferon-γ (IFN- γ), two of the most important proinflammatory cytokines[25]. Catabolites of tryptophan produced by MSCs, are also bioactive, since they act suppressing both CD4+ and CD8+ T lymphocyte subtypes activation.

In brief, cumulative results show that any immunosurveillance cell coming into the range of MSCs will be suppressed. This feature grants MSCs several abilities, such as escaping host immuno-recognition, inhibiting immunosurveillance at the injury site and preventing autoimmune events to estabilish. Therefore, alloreactivity doesn't seem to be a major problem for MSCs and their addition to a bone graft should protect it from the host immune system, enhancing its survival probabilities.

[25] Aggarwal and Pittenger, 2005

PRE-CLINICAL STUDIES, CLINICAL TRIALS AND CURRENT APPLICATIONS OF ADIPOSE-DERIVED MSCs

As described above, therapeutic uses of ASCs are supported by two important characteristics of this cell lineage: regenerative properties and immunomodulatory activity. To date, proposed employments for ASCs in tissue repair and regeneration are quite impressive and can be listed following clinical application criteria.

1) Musculoskeletal tissues regeneration;
2) myocardial infarction;
3) applications based on ASCs immunomodulatory properties;
4) gastrointestinal diseases;
5) urogenital system disorders;
6) nervous system diseases;
7) wound healing;
8) plastic surgery and tissue reconstruction;
9) other clinical trials.

1) Musculoskeletal tissue regeneration

Considering the adipose tissue mesodermal origin, application of ASCs to bone and cartilage defects is obvious, along with their uses in tendon and invertebral disk repair.

Succesful outcomes in pre-clinical researches include:

a) repairing of calvarial defects, studied both in rat ([26]) and rabbit models (Dudas et al., 2006);

b) repairing of rats cleft palatal bone defects[27];

c) repairing of rabbits tibia proximal epiphysis[28];

d) repairing of mice cartilage defects using a human ASCs (hASCs) tissue-engineered cartilage[29];

e) primary tendon repair in an in vivo tendon injury model[30];

f) intervertebral disc regeneration in small animals model, such as rats and rabbits and in larger animal models, such as goat and canine ;

g) facilitation of spine fusion in rats using allogeneic ASCs isolated both from rat and from human adipose tissue.

For what it concerns data on humans, to date licterature decscribes two important case reports and and one ongoing clinical trial (NCT01218945).

The first is a report of a 7-year-old girl suffering from a widespread calvarial defects after severe head injury[31]. Due to the limited amount of autologous cancellous bone, autologous ASCs were purified and applied to the calvarial defects toghether with autologous fibrin glue. Three months after the reconstruction, CT-scan showed new bone formation and almost complete calvarial continuity.

[26] Cowan et al., 2004; Yoon et al., 2007
[27] Conejero et al., 2006
[28] de Girolamo et al., 2010
[29] Dragoo et al., 2003
[30] Uysal and Mizuno, 2009
[31] Lendeckel et al., 2004

The second reports the orbital floor reconstruction of a 65-year-old male patient who had undergone a hemimaxillectomy due to a large keratocyst. The large defect was reconstruct with a titanium cage, filled with autologous ASCs and betaTCP, that was previously inserted for 6 months in a pouch prepared in the patient's left rectus abdominis muscle. Success of this reconstruction is mainly to ascribe both to bony neotissue and good vascularization of the titanium scaffold; this result also indicates that ASCs promote intense neovascularization, a crucial feature for grafts survival.

The clinical trial number NCT01218945 concerns the development of engineered synthetic bone grafts, preloaded with hASCs, to repair large osseous defects.

2) Myocardial infarction

Numerous studies in animal models have investigated the ASCs potential for treating myocardial infarctions and chronic heart failure[32]. ASCs mainly exert their myocardial regenerative effect through secretion of trophic soluble factors[33]. Again, paracrine activity seems to play a key role in ASCs-mediated therapeutic properties.

In humans, there are two ongoing phase I clinical research studies (NCT00442806 and NCT00426868).

[32] Hwangbo et al, 2010; Mazo et al., 2010; Bai et al., 2010; Valina et al., 2007
[33] Bai et al., 2010

3) Applications based on ASCs immunomodulatory properties

The capacity of ASCs to regulate a wide spectrum of inflammatory mediators, offers a precious therapeutic tool to treat several clinical conditions needing pharmacological immunosuppresion.

Pre-clinical studies include:

a) treating of mice experimental arthritis with hASCs

b) treating of mice experimental allergic rhinitis with allogenic mASCs;

c) anti-rejection activity in organ transplantation; in a rat liver transplantation model, allogeneic ASCs significantly alleviated acute rejection. This field of application holds great promises for the future of MSCs cell lineages, however, to date, sudies are limited to animal models;

In humans, an encouraging result comes from a study reporting allogeneic infusion of hASCs in six patients who have developed chronic and extensive graft versus host disease (GvHD), after haematopoietic stem cell transplantation[34]. In addition, allogeneic infusion of hASCs has also been approved to be used for the same application in an ongoing phase II clinical trial (NCT01222039).

4) Gastrointestinal diseases

hASCs have also been shown to be a valuable opportunity to treat patients with intractable enterocutaneous[35], perianal and rectovaginal fistulas[36], as a result of Crohn's disease. Four related clinical trials are reported:

[34] Song et al., 2007

[35] Garcia-Olmo et al., 2009A

a) safety and efficacy study of autologous cultured hASC for the Crohn's fistula, phase I, completed (NCT00992485);

b) safety and efficacy study of autologous cultured hASC for the Crohn's fistula, phase II, ongoing (NCT01011244);

c)allogenic hASCs derived from lipoaspirates for the treatment of recto-vaginal fistulas associated to Crohn`s disease, phase I and II, ongoing (NCT00999115);

d)treatment of fistulous Crohn's disease by implant of autologous hASCs, phase I and II, ongoing (NCT01157650)

Interestingly, no pre-clinical studies are available for the same indications.

5) Urogenital system disorder

ASCs regenerative properties have also been applied in several urology preclinical researches:

a) treatment of rats stress urinary incontinence [37];

b) rats and rabbits bladder reconstruction;

c) treatment of erectile dysfunction in obese type 2 diabetic;

In addition, one case report has been recently published, regarding two patients that receive periurethral injection of autologous ASCs for urinary incontinence, due to post-radical prostatectomy (Yamamoto et al., 2010). This prelminary study showed that periurethral injection of autologous ASCs is a safe and feasible treatment modality for stress urinary incontinence in humans.

[36] Garcìa-Olmo et al., 2010

[37] Jack et al., 2005; Lin et al., 2010

6) Nervous system diseases

As shown by pre-clinical results, ASCs trophic activity improves nervous system's cell replacement and tissue regeneration. Proposed field of application include:

a) improving of brain recovery in rat stroke models -hASCs-;
b) improving of motor function in rat models of spinal cord injury -autologous rASCs-;
c) repairing of injured rats peripheral nerves –hASCs-.

In human, a safety/efficacy phase I and II clinical study is evaluating the feasibility of regenerative therapy with autologous ASC, administered intravenously, in patients with secondary progressive multiple sclerosis who do not respond to regular treatments (NCT01056471).

7) Wound healing

Therapeutic potential of ASCs in wound healing has also been investigated.

In rats mitomycin C-treated healing-impaired wounds, local application of autologous ASCs can induce significant wound healing acceleration[38].

Clinical outcome potential was also confirmed in humans. Twenty patients being treated for the side effect of radiotherapy, with severe symtpoms, received autologous ASCs via repeated hypoinvasive computer-assisted injections; this

[38] Nambu et al., 2007

clinical approach led to a systematic improvement or remission of symptoms in all evaluated patients[39].

8) plastic surgery and tissue reconstruction

Engineer of adipose tissue finds one of its major expressions in plastic surgery and in tissue reconstruction fields. Four clinical trials are currently reported:

a) phaseIV post-marketing study evaluating the transplantation of autologous fat enriched with ASCs, in patients with functional and cosmetic breast deformities post lumpectomy (NCT00616135);

b) completed phase II and III clinical trials evaluating the safety and efficacy of autologous adipocytes and ASCs, differentiated towards the adipocytes phenotype, to treat depressed scars (NCT00992147);

c) phase I study determining the safety of the autologous ASCs transplantation in the treatment of lipodystrophies (NCT00715546);

d) completed phase III clinical trial investigating safety and efficacy of autologous ASCs for the closure of perianal fistulas in patients without Crohn´s disease (NCT00475410).

9) Other clinical trials

[39]Rigotti et al., 2007

For what it concerns ASCs-based ongoing clinical trials, others four human applications are currently under investigation:

a) phase I and II clinical studies determining whether intravenous administration of autologous adipose ASCs is safe and beneficial in patients with type 1 diabetes (NCT00703599);

b) phase I and II trials determining whether intravenous administration of autologous ASCs would account a benefit in the types 2 diabetics management (NCT00703612);

c) completed phase III clinical trial investigating safety and efficacy of autologous ASCs for the closure of perianal fistulas, in patients without Crohn´s disease (NCT00475410);

d) phase I and II studies evaluating safety and feasibility of regenerative therapy with autologous ASCs, administered intramusculary, in patients with critical leg ischemia (NCT01211028).

SAFETY CONCERNS

The use of adult MSCs -including ASCs- in cell-based therapies is considered safer and more functional than use of either hESCs and iPSs. In fact, MCSs are immunocompatible and don't require genetic manipulation; moreover, their clinical employment doesn't elicit any ethical controversy.

ASCs are known to undergo malignant transformation during protracted culture *in vitro* (20-30 passages); however, for clinical applications, it is unlekely that there will be a need for cultures longer than one passage.

Finally, no adverse and rejection reactions were reported in pre-clinical and clinical trials, thus confirming the high safety rate of ASCs.

Finally it can ba considered that MSCs are the major candidates for the future of regenerative therapies. Among several proposed putative sources of MSCs, adipose tissue has been proven to be the most promising because of three intrinsinc features: high yield of stem cells, avalaibility and easy harvesting. In addition, it has been demonstrated the ability of ASCs to suppress specific aspects of immune system, toghether with pre-clinical and clinical studies reporting no rejection -or adverse effect- for allogeneic treatment. The possibility to use unmatched allogeneic ASCs implies that a single lot of cells, derived from one donor, could be transplanted into multiple patients. There are two consequent advantages in that: reduction of the quality control costs and benefit for treated patients, that would be always transplanted with young and healthy selected cells.

Abilities of adult ASCs in promoting bone formation and grafts survival are well established. Even though, focus of investigations surrounding ASCs applications in spine fusion is still limited[40]. However, the physiological characteristics of ASCs indicate that this cell lineage possesses exciting potentials in the stem cell-based regenerative therapies. For this reason, optimization of both cell growth and choice of scaffold will offer succesful surgical outcomes in several orthopaedic applications.

[40] Lopez et al., 2009; Hsu et al., 2008

Chapter 2

Cell separation

Overview on cell separation

Cell separation is a powerful tool, which is widely used in many strands of biological and biomedical research and in clinical therapy. For research, the ability to sort cells into distinct populations enables the study of individual cell types isolated from a heterogeneous starting population without (or with greatly reduced) contamination from other cell types. This technology underpins many discoveries in cell biology and is further enabling research in areas as diverse as regenerative medicine, cancer therapy and HIV pathogenesis.

In terms of clinical usage, therapeutic cell separation allows for the introduction of enriched cell populations to a patient with a clinical need for those cells, for example, separation of leukocytes by aphaeresis or enrichment of haematopoietic

stem cells by immunomagnetic separation[41] [42]. It also enables the enumeration of cells within an individual's blood system and can aid repopulation of the immune system, for example, in multiple sclerosis patients who have undergone immunoablation treatment.

Currently, most regenerative treatments based on cell separation are restricted to tissues such as blood and bone marrow[43]. Recently, however, advances in stem cell therapy, tissue engineering and regenerative medicine are showing the potential for clinical cell-based therapies using cells derived from a variety of tissues, such as adipose and intestine. The use of highly selective cell separation procedures in clinical cell-based treatments has the potential to improve the quality of repair and the subsequent clinical outcome. Because of this potential, there is an increasing usage of these methodologies in the fields of tissue engineering and regenerative medicine, which has resulted in an increasing number of researchers using, or wanting to use, cell separation technologies. These researchers are drawn from a diverse range of backgrounds, not all of whom are necessarily based in biology. Indeed, the increasing demand for cell separation in multiple disciplinary research fields is not restricted to tissue engineering and regenerative medicine; cell sorting is also being used in many other areas such as biochemistry, electrical engineering, physics and materials science.

A multitude of cell separation techniques currently available to researchers are based on three core themes: density, adherence and antibody binding, with many points of crossover between these different themes. New techniques incorporating microfluidics combined with a variety of cellular properties are also in development.

---

[41] Handgretinger R, Lang P, Schumm M, et al. *Bone Marrow Transplant* 1998; 21: 987–993

[42] To LB, Haylock D, Simmons PJ, et al. The biology and clini-cal uses of blood stem cells. *Blood* 1997; 89: 2233–2258

[43] Stamm C, Westphal B, Kleine H-D, et al. *Lancet* 2003; 361: 45–46

Despite the differences between different cell separation techniques, they share common problems and pitfalls, which can at best hinder research progress and at worst give rise to erroneous data. Many of these technical problems and pitfalls are only applicable to certain techniques, whereas others are universal regardless of the method of separation. Other difficulties can arise in the experimental planning stage, where there can be a lack of understanding in identifying appropriate controls. Finally, there is a potential lack of clarity in the terminology used around cell separation methods, which can lead to confusion and a misunderstanding of the analytical measures required.

This review is written taking cognisance of the diversity of backgrounds and expertise of those researchers wishing to use cell sorting methods. The aim is not to produce a detailed step-by-step guide for each methodology but to offer potential solutions when common difficulties arise and provide clarity in areas of ambiguity related to experimental preparation and terminology.

Cell separation techniques

A large variety of cell separation methods are currently commercially available, these are predominantly based on three methodologies: adherence, density and antibody binding. New techniques are being developed that utilise microfluidic technologies and take advantage of a variety of cellular properties such as elasticity in response to acoustic waves and membrane polarisation in a non-uniform electric field[44]. [45] However, these techniques are mostly still experimental and not yet available commercially for research. The choice of separation method

[44] Petersson F, Åberg L, Swärd-Nilsson A-M, et al. *Anal Chem* 2007; 79: 5117–5123
[45] Hu X, Bessette PH, Qian J, et al.*Proc Natl Acad Sci U S A* 2005; 102: 15757–15761.

depends upon a variety of factors, and each methodology has benefits and drawbacks that affect its applicability in a given situation. In this section, we will briefly outline the three overall methodologies with specific examples of each.

Adherence

Techniques that utilise cellular adherence are some of the most simple methods used for cell separation and are routinely used when isolating cells from digested or explanted primary tissues (Figure 1.2). An example of simple cell separation by adherence is the isolation of dental pulp stromal cells from whole digested dental pulp. In this technique, enzymatically digested dental pulp is filtered and plated directly onto tissue culture plastic, and following a period of culture, the adherent stromal cells are passaged.[46] This technique benefits from being very simple and cheap, but it is not at all specific and relies on the cells of interest adhering and in some instances rapidly proliferating to outcompete other adherent cells in the suspension, such as neurons and monocytes. Adherence can also take time leading to some uncertainty as to the success of a separation. Recently, techniques based on cell adherence, such as differential binding of cells to polymer brushes of varying lengths, grafted to glass surfaces, have been developed and these are currently being refined.However, despite this progress, current uses of adherence sorting are mostly only applicable when cell purity is not of concern and isolation of various subpopulations is not required.

[46] Gronthos S, Mankani M, Brahim J, et al. *Proc Natl Acad Sci U S A* 2000; 97: 13625–13630

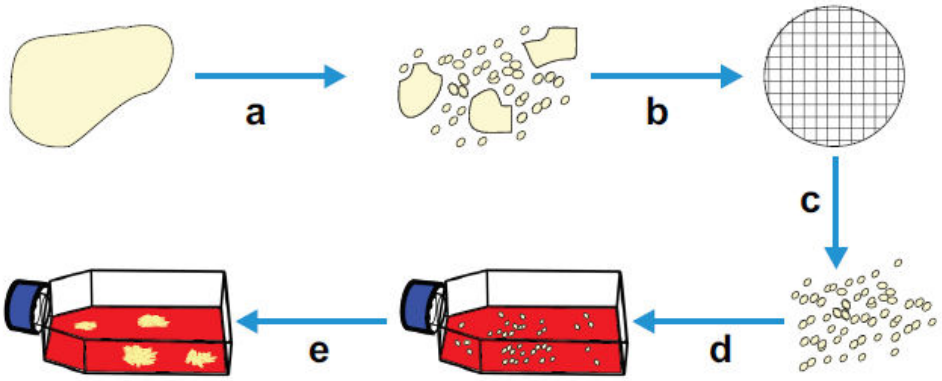


Figure 1.2. Diagram detailing cell separation by plastic adherence. (a) Whole tissue is disrupted into a cell suspension by enzymatic or mechanical means or a combination of both (separations of blood or bone marrow aspirate do not require this step). (b) Following disruption, the cells can be passed through a filter to remove cell clumps (c) giving a single-cell suspension, which will be added to (d) an adherent surface, and after a period of culture, (e) adherent cells can be observed.

Density

Density-based techniques are now mostly based on the use of centrifugation, although historically sedimentation-based methods have been employed[47] .Techniques based on centrifugation are commonly used in many laboratories and are also routinely used clinically. The ability to sort large numbers of cells based on their density, relative to a graduated separation medium (usually sugar based), makes these techniques particularly applicable for separations involving the use of blood (Figure 1.3), which contains 4 × 109 to 6.5 × 109 cells/mL. Indeed, the most

[47] Liu W, Hou Y, Chen H, et al. *Proteomics* 2011; 11: 3556–3564

commonly used clinical cell separation method is aphaeresis of whole blood to isolate mononuclear cells for treatment of a variety of conditions, including leukaemia[48]. However, despite the large-scale use of density-based methods, there are still problems with specificity as the differing densities of different cell populations are, in some instances, not large enough to be able to separate out individual cell types. These problems can be overcome by performing repeated centrifugations using differing concentrations of centrifugation medium and differing angular velocities. By using these techniques, it is possible to isolate different cell types from a complex mix, including disrupted solid tissues (Figure 1.4) such as mouse liver. However, although technically feasible, this is still challenging to perform with high specificity. As such, centrifugation methods are generally used if specificity is not absolutely necessary, as in aphaeresis, or as a pre-enrichment stage to remove cells like red blood cells and platelets.

Another density-based method used in laboratory separations is rosetting, which works as a combination between antibody binding and density methods. In this method, unwanted cells are labelled with antibodies that subsequently form complexes with erythrocytes, creating immunorosettes that are much denser than the mononuclear cells of interest. Following centrifugation, these rosettes, containing the labelled unwanted cells, pellet with erythrocytes leaving purified target cells in the mononuclear cell phase.21

[48] Buckner D, Graw RG, Eisel RJ, et al. *Blood* 1969; 33: 353–369

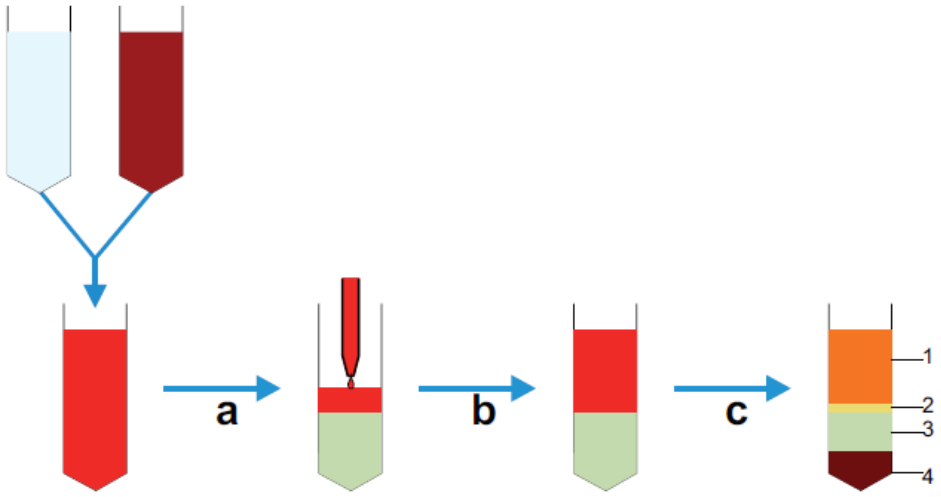


Figure 1.3 : Diagram detailing whole blood cell separation by density gradient centrifugation. (a) Initially, whole blood is diluted with saline buffer, and (b) this is then carefully layered on top of the centrifugation medium contained in a conical tube avoiding any mixing of the two phases. (c) Following centrifugation, at the appropriate velocity without braking, distinct phases can be observed; 1 – plasma, 2 – interphase containing mononuclear cells, 3 – centrifugation medium and 4 – erythrocytes and granulocytes; cells can then be aspirated from the interphase.

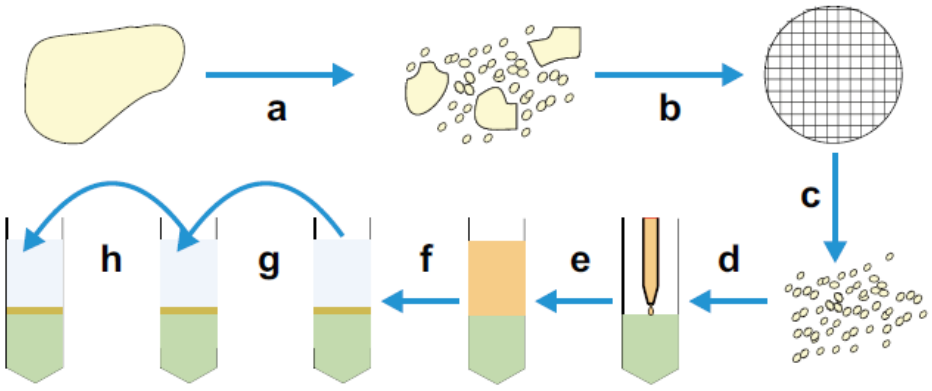


Figure 1.4: Diagram showing separation of solid tissue–derived cells by density gradient centrifugation. Tissues are (a) dissociated and (b) filtered to give (c) a

single-cell suspension. (d) This suspension is carefully layered over a centrifugation medium avoiding mixing to give (e) two distinct phases, which can then be centrifuged to give (f) a cell-rich interphase between the centrifugation medium and the cell suspension buffer. (g and h) It is possible to isolate different cell fractions by removing cells from the supernatant or the interphase and then recentrifuging them at different concentrations of centrifugation medium and angular velocities until the desired fractions are obtained.

Methods that sort cells by density are useful techniques to employ when working with tissues that contain a large number of unwanted cells, for example, blood, bone marrow and adipose tissue. This can be either for the isolation of a heterogeneous mix of cells, which can then be used experimentally, or as a pre-enrichment step prior to sorting by other methods.

Antibody binding

Antibody-binding methods generally refer to the commonly used techniques of fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS)[49] [50].[51] Both technologies utilise the same cellular properties for separation, namely, cell surface antigens against which antibodies are raised. FACS separation relies on the conjugation of fluorescent labels to these antibodies, whereas MACS uses conjugation to iron oxide containing microbeads. Following binding of conjugated antibodies, FACS and MACS proceed down different routes. FACS separation is achieved by laser excitation of the bound fluorophores, with

[49] Bonner WA, Sweet RG, Hulett HR, et al. *Rev Sci Instrum* 1972; 43: 404–409
[50] Miltenyi S, Müller W, Weichel W, et al. *Cytometry* 1990; 11: 231–238
[51] Rembaum A, Yen RCK, Kempner DH, et al. *J Immunol Methods* 1982; 52: 341–351.

excitation above a threshold level signalling the corresponding cell to be separated (Figure 1.5).

MACS requires the cells to be placed in a magnetic field; unlabelled cells are eluted, and labelled cells are retained in the field until they are removed from the magnet, giving the separated populations (Figure 1.6).

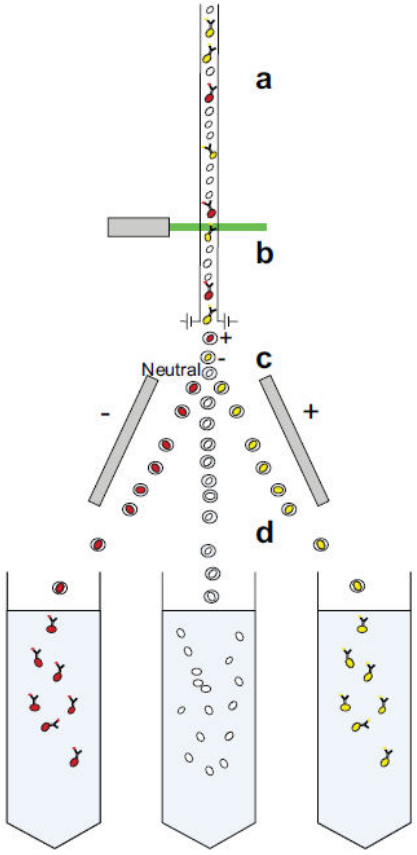


Figure 1.5 Diagram showing cell separation by FACS. Fluorescently labelled single cells from solid or fluid tissues, filtered to remove cell aggregates, are channelled to give a continuous stream of individual cells; (b) these cells then pass through a light source or laser, and the signature of each cell is detected. From this detection, the cells will be determined to be above or below a designated threshold value, and it is decided whether to collect or not collect each cell. (c) This is achieved by electrically charging the droplet

each cell is contained within and (d) then by passing it through charged deflector plates that deflect the cells to the appropriate collection tubes.

FACS: fluorescence-activated cell sorting.

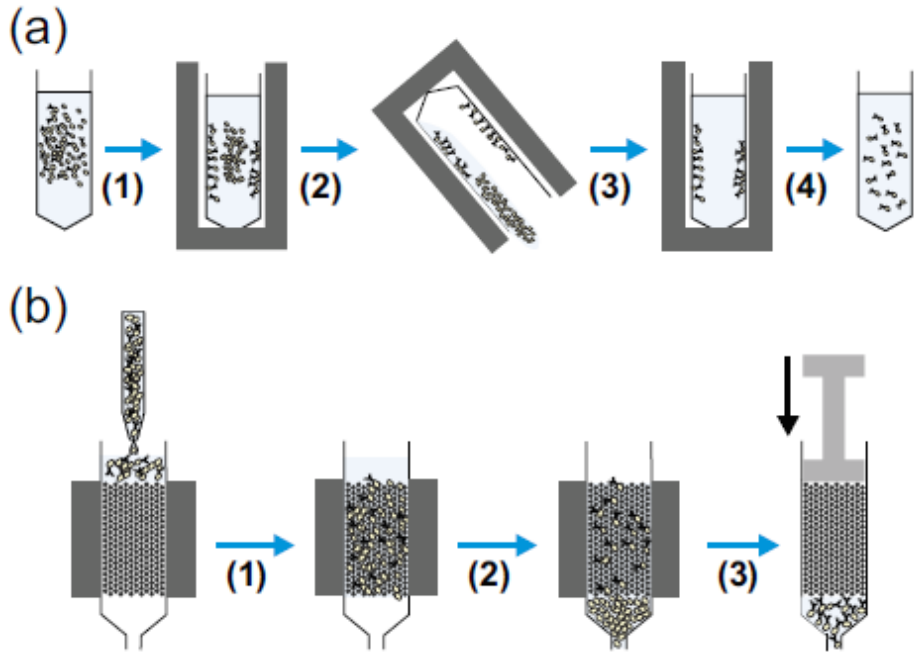


Figure 1.6: Diagrams showing the common methods used for magnetic cell separation. (a) Tube-based separation where a magnetically labelled cell suspension held in a conical tube is placed in a (1) magnet causing movement of labelled cells to the sides of the tube towards the magnet. This tube is then (2) inverted (or aspirated), allowing removal of the non-labelled cells before (3) resuspension of the labelled cells and removal from the magnet giving (4) a dispersed suspension of labelled target cells. (b) Column-based separation where a magnetically labelled cell suspension is injected into a column held within a magnet, (1) cells then flow through the column and (2) labelled cells are retained, whereas unlabelled cells are washed out. (3) Following the removal of unlabelled cells, the column is removed from the magnet, and suspension buffer is forced through the column by plunger giving labelled target cells in suspension.

As such, a key difference between MACS and FACS is that MACS can be seen as a bulk method, there is no individual cell analysis, and magnetically tagged cells

are retained and non-tagged cells are eluted. FACS, however, analyses each individual cell, which can be tagged with multiple antibodies, whereas MACS is restricted to individual markers (although some kits use enzymatic removal of the microbeads, allowing the cells to be relabelled with a subsequent antibody). This individual cell analysis means that while FACS can be more specific, it is significantly slower than MACS. Sorting that takes several hours by FACS can be achieved in less than 1 h by MACS.

There are other techniques, in addition to FACS and MACS, that utilise antibody binding to enable cell separation, an example of which is rosetting as previously mentioned. However, this is a relatively old technique, and there are many new technologies being developed, which use antibody or cell–ligand binding as the basis for separation. For example, antibodies, immobilised to polymer surfaces, have been used in a microfluidic system to capture circulating tumour cells from whole blood with subsequent release and enumeration. Columns have also been developed with antibody-immobilised surfaces to enrich osteoblastic cells based on CD34 binding. Polymer cryogels with large interconnected pores and surface-immobilised protein A ligands have been used to isolate antibody-labelled CD34+ umbilical cord blood cells in an affinity chromatography–based separation.[52] Other methods in development include magnetophoresis, DNA aptamer binding[53] and aqueous phase partitioning[54]. However, despite the variety of antibody-based methods, for the purposes of this review, FACS and MACS will be focussed on due to the experimental nature of these newer techniques.

Antibody-based methods of separation are currently the gold standard for the selection of individual cell populations, and both FACS and MACS can be used to

[52] Kumar A and Srivastava A.. *Nat Protoc* 2010; 5: 1737–1747
[53] Xu Y, Phillips JA, Yan JL, et al *Anal Chem* 2009; 81: 7436–7442
[54] Sousa AF, Andrade PZ, Pirzgalska RM, et al. *Biotechnol Lett* 2011; 33: 2373–2377

isolate cell populations to high purity. Despite this, there are still some problems with FACS and MACS such as the reliance on cell surface markers, which, for most researchers, limits separations to those markers for which antibodies are commercially available. It can also cause problems if the cell type of interest does not have unique markers, making the isolation of a homogeneous population difficult. For example, mesenchymal stem cells (MSCs) express markers associated with many other cell types such as CD90, which is also expressed by primitive haematopoietic stem cells. In addition, the isolation of a viable homogeneous population of cells that contain a unique intracellular marker can also be problematic, as the permeabilisation steps required to stain the marker can damage cell membranes leading to cell death.

Lab-on-a-chip methods

In addition to the traditionally used methodologies for cell separation are several new lab-on-a-chip techniques that operate on a microfluidic scale and utilise a multitude of cellular characteristics to isolate different cell populations in a label-free manner. These techniques are mostly still in the experimental stage, but their development demonstrates the variety of possible ways to separate cells, and they are extensively reviewed by Gossett et al.[55] Examples of label-free separation are the use of micro-scale filters or pillars that separate cells based upon size and membrane deformability, as larger cells are prevented from navigating through the filter leading to cell separation.[56] Field flow fractionation (FFF) can be used to separate cells along the length of a microfluidic channel by a combination of the parabolic flow within the channel and an external field, such as an electric field or

[55] Gossett DR, Weaver WM, Mach AJ, et al.. *Anal Bioanal Chem* 2010; 397: 3249–3267.

[56] Ji HM, Samper V, Chen Y, et al. *Biomed Microdevices* 2008; 10: 251–257.

gravity. With FFF, particles that are more greatly affected by the external field are forced closer to the channel wall, which is moving more slowly than the centre of the channel and contains more weakly affected particles. Therefore, cell separation occurs because of the effect of the force on the cells and the speed of elution based on the cells' location in the microfluidic channel.[57] Acoustophoresis separates cells based on membrane deformation or elasticity and occurs when a high-pressure sound wave interacts with a cell. This interaction can cause membrane deformation to differing degrees based on the cell's density and size and leads to the cells being positioned in different parts of the microfluidic channel and therefore able to be separated. Dielectrophoresis can lead to cell separation due to the differential polarisation of particles within a non-uniform electric field. This dipole effect depends on factors such as size and protein content and leads to the attraction or repulsion of the cell away from or towards an electrode. Due to differences in these factors between different cells, it is therefore possible to exert different effects on different cell types within the same field and allow for cell separation.

Label-free lab-on-a-chip isolation methods have great potential to improve cell sorting methods both in a research environment and clinically. However, there are still potential problems associated with these techniques, many of which are general cell sorting problems, which can be applied to the commonly used techniques such as cell clusters, and others that are technique specific. One of the largest problems these techniques currently face is resolving the differences between cell types; for example, with dielectrophoresis, it can be difficult to discern the differences between target and non-target cells. However, perhaps the greatest

[57] . Vykoukal J, Vykoukal DM, Freyberg S, et al. *Lab Chip* 2008; 8: 1386–1393.

challenge these techniques face is showing great enough efficacy while overcoming the challenges associated with currently used methods.

Overall, the choice of cell separation methodology is very much dependent upon the initial cell source, the characteristics of the desired cell type and its required purity. Adhesion-based techniques are useful if there is little requirement other than the isolation of adherent cells, and the cell of interest will, if necessary, outcompete other cell types. Centrifugation techniques are useful when dealing with samples with large cell numbers, such as blood, but where specificity is not essential, and are also useful as a pre-enrichment step prior to other separation methods. Antibody-mediated separation methods are the gold standard techniques currently available as they can be used to isolate specific cell populations. However, speed can be an issue, as can costs. Potentially, lab-on-a-chip methods will overcome some of the limitations in the currently used techniques, but, as yet, these are experimental and not accessible to the majority of the researchers performing cell sorting.

Clinical cell therapy

The majority of separations currently performed for clinical cell therapy use cells isolated from tissues such as bone marrow and blood. These separations isolate the mononuclear cells, including the stem cell fraction, and can be used to recapitulate the haematopoietic system of a patient suffering from, for example, chronic myeloid leukaemia, following immune ablation therapy. These separations mostly utilise systems based on centrifugation, such as aphaeresis, as these technologies allow for the isolation of the large numbers of mononuclear cells needed for cell transplantation relatively quickly. MACS can also be used for cell

therapy, and the clinically approved MACS-based systems use the same technology as research-grade magnetic sorting; however, these systems are closed and use reagents and fluidic tubing produced under good manufacturing practice (GMP) conditions.[58] Use of MACS for clinical cell sorting allows for greater specificity than can be achieved by centrifugation; however, per patient, MACS is more expensive than aphaeresis, and so it is used in circumstances where specificity of the isolated cells is important.

Standard FACS-based systems are not in clinical use for cell therapy, although some flow cytometers can be used for clinical diagnostics[59]. This is in part due to the difficulty in developing single-use sterile fluidics, the possibility of cross-contamination should multiuse fluidics be employed and problems with batch-to-batch consistency. There are currently methods utilising closed system optical separation in development, but these are not yet in widespread clinical usage.

Clinical cell separation is an established field, but it has strict requirements, and there are challenges and difficulties to overcome. The major requirement is to ensure that a consistent, sterile cell population is isolated. Microbial contamination of cell separation products could lead to the infection of the recipient patient, who, in many instances, will be immunocompromised and unable to fight the infection. It is therefore imperative that clinical cell separation products are produced under strict GMP conditions with stringent batch testing. Consistency of the isolated cell population is also very important so as to ensure that the recipient receives the required cell transplant. In addition, rigorous tissue typing should be performed prior to transplantation to avoid human leukocyte antigen (HLA) mismatch and prevent problems such as graft-versus-host disease.

[58] Lang P, Schumm M, Taylor G, et al. 1999; 24: 583–589.
[59] Brown M and Wittwer C. *Clin Chem* 2000; 46: 1221–1229

At this time, the major challenge for clinical cell separation is the robust isolation of rare cell populations with multiple surface markers from a large initial pool of cells. Currently, technologies based on centrifugation allow for the isolation of cells from a large initial cell number, and technologies based on MACS can isolate specific populations of cells; however, these technologies use single markers meaning that cells of interest with two or more markers cannot be specifically isolated. Development of high-speed optical cell sorters holds great promise, as these systems could have the speed of an MACS-based system, but with the specificity of an FACS system allowing for more than one parameter to be selected.

Considerations for experimental design

Initial planning and design is key for any experimental strategy, including cell separation, where many factors must first be considered. These factors impact different stages of the separation procedure, but all share a basic set of preliminary requirements. These are the need for a detailed understanding of the cell and tissue types of interest, knowledge of the potential techniques available and the ability to select the correct methodology to yield the desired cell population.

The reason for this required level of understanding is that one cell separation method may be more suitable than another for achieving a given outcome, and different cells react differently to the same conditions. Current methods for cell separation generally offer a balance between purity and recovery. It is therefore important that the separation protocol is designed with this in mind and tailored to suit the desired outcome. For example, if a large number of cells are required, then percentage enrichment may need to be sacrificed; alternatively, for a highly

enriched population, the trade-off may be low numbers recovered. Factors to be considered when designing a cell separation strategy are discussed below.

Cost

Cost is a design constraint that is relevant to most separation experiments. Cell separation can be a potentially expensive technology depending on the strategy selected. It may therefore be important to devise a strategy that is not prohibitively expensive by employing cost-saving measures. For example, FACS is a very accurate technique, but it can be slow when sorting rare cells from whole blood, and this consequently increases the running time on the instrument and thus the expense. A way of reducing this time would be to perform an initial erythrocyte lysis step or density gradient centrifugation to remove the erythrocytes, leaving only the mononuclear cells to sort.[60] Pretreatment of a sample can thus reduce overall cost and should be considered where cost is an issue.

Methodological difficulties

There are several key technical considerations that must be taken into account before performing a successful cell separation, some of which are universally applicable, while others are more specific to immunomagnetic and immuno-fluorescent cell separation. Figure 6 gives an overview of potential technical problems at each stage during the separation process.

The more universal considerations relate to the quality of the cells, which are being separated, and specifically to the cell isolation process. Antibody-mediated separations also have considerations relating to antibody binding. There can also be specific idiosyncratic problems associated with different commercially available

[60] Bøyum A. *Scand J Immunol* 1976; 5: (Suppl. 5): 9–15

cell separation products such as incubation temperature and supernatant removal, but it is not within the remit of this review to discuss these. Any specific technical issues is best dealt with by the company responsible for the product.

Cell isolation and preparation are essential prerequisites when sorting cells but can be the cause of many technical difficulties when resulting suspensions contain clusters of cells and/or a high proportion of dead cells. For the purposes of this review, we are defining a cell cluster as an association of two or more cells. Cell clusters can arise when working with both solid tissue- and blood-derived cells due to incomplete dissociation or post-dissociative association/aggregation. The presence of cell clusters can result in reduction in isolated cell purity due to co-isolation of non-target cells that are conjoined with the cells of interest or loss of target cells due to their binding with cells that are removed from the suspension as part of the separation process.

Currently available strategies for cell separation can yield highly enriched cell suspensions. However, there are potential problems that can impair the overall quality of the separation, and these need to be recognised by the increasingly interdisciplinary user base and addressed where they arise. In addition, experimental planning and terminology need to be carefully considered.

In the initial experimental design and planning stages, it is important to understand what outcomes are going to be assessed, that is, how are purity, recovery and viability being measured? It is important to identify the characteristic which purity is being measured against, which population the recovered cells are being compared to and which measure of viability is being assessed. If these terminological ambiguities are defined prior to beginning the experimental regimen, it can make identifying technical problems easier.

A thorough knowledge of the cell suspension and the cell type to be isolated can vastly improve the quality of a separation. This is particularly important for cell separation based on antibody binding. It is also important to collect samples at key points during the separation process so that the efficiency of the separation can be assessed. This point is related to the terminological considerations, as these data are required to determine the purity, recovery and viability. Perhaps the most important preparatory step prior to separation is the storage of the starting tissue. The key aspect with this is speed of tissue processing, with dissociation and cell separation immediately following tissue excision being greatly preferred. If this is not possible, then various means can be employed to reduce tissue necrosis, the most important of which is temperature.

Technically, there are several factors that can compromise the quality of a cell separation and subsequently the overall data acquired. These factors can be distilled down to two main problems: clusters of cells and false-positive cell sorting. Both of these problems have multiple causes, some of which overlap. The general problems are incomplete tissue digestion, re-establishment of cell–cell contacts, release of DNA by dying cells, non-specific antibody labelling of dead cells and non-specific antibody binding to the FcR. None of these technical problems are insurmountable, but they can lead to significant problems without knowledge and awareness of the issues together with appropriate measures taken to address them.

Overall, it is hoped that this review clarifies terminology, provides guidance to experimental set-up and gives reasons for and solutions to potential problems that can arise during the process of cell separation. We hope that we have achieved our aim of providing the user with an understanding of why certain terminology is

used and what it means, why certain aspects of planning and set-up are key to successful separations and what the main technical difficulties that can arise during the process are and how they can be resolved.

FIELD FLOW FRACTIONATION

Novel tagless separation techniques have emerged as alternatives to current methodologies for stem cells isolation. The most reported examples of these innovative technologies are dielectrophoresis (DEP), aqueous two phase systems and field flow fractionation (FFF). All of these methodologies do not require the use of a molecular tag, eliminating incubation times and often the label removal or detaching step. A relevant feature of this group of methods is their feasibility to be implemented at microscale (lab-on-chip).

FFF is a chromatographic-like, soft impact separation method that performs partition based on mass, size, charge, density, shape, and rigidity. Separation is achieved within a capillary channel by the combined action of a mobile phase in laminar flow and a field that is applied perpendicularly to the flow. According to their physical characteristics, cells are distributed at different positions within the flow profile resulting in different elution times, and fractions can be collected separately.[61] Different types of field have been used in FFF: gravitational field flow (GrFFF), centrifugal sedimentation field flow (SdFFF) and dielectrophoretic field

[61] A. Lucas, F. Lepage, P. Cardot (2000) Cell separations. In M. E. Schimpf, K. Caldwell & J. C. Giddings (Eds.), Field-flow fractionation hand-book, chapter 29 (pp. 471–486). New York: Wiley-Interscience.

flow (DEP).[13] DEP-GrFFF has been also scaled up to process up to 1 million cells per run, which can be compared to the performance of the FACS but with significant lower cost.

Elution mode in FFF

Normal mode

The normal FFF mode drives the elution of macromolecules and submicrometer particles. As the macromolecules or particles that constitute the sample are driven by the field toward the accumulation wall, their concentration increases with decreasing distance from the wall (Figure 2.1a). This creates a concentration gradient that causes sample diffusion away from the wall. When these two opposite transport processes balance, the sample cloud reaches a characteristic average elevation from the wall. The lower the molar mass or size of the sample component, the greater the component cloud elevation, the deeper the cloud penetration into the faster streamlines of the parabolic flow profile and the shorter the time required by the component to exit the channel. Retention time in normal FFF is therefore shorter for lower molar mass or size.

Steric and hyperlayer mode

If the sample components are micron-sized particles, their diffusion away from the wall is negligible. Particles are in fact driven by the field directly to the accumulation

wall. Particles of a given size form a thin layer of a given thickness, hugging the wall.

Larger particles form thicker layers that penetrate into faster streamlines of the parabolic flow profile, and they are eluted more rapidly than smaller particles. This is just the opposite of normal mode elution: it is then referred to as a reversed mode. This elution mode is in fact governed by the physical (steric) barrier of the accumulation wall, so is called ‘steric’ (Figure 2.1b). Retention in steric FFF then depends only on particle size. During elution, however, the micron-sized particles make very little contact with the wall. Instead, their moves toward the wall are opposed by mobile phase flow-induced lift forces (Figure Ic, green arrows). When particles are driven from the wall by a distance that is greater than their diameter, the retention mode is called hyperlayer (Figure 2.1c). Retention in hyperlayer mode is still reversed with respect to particle size but it also depends on thevarious physical features of the particles, which will have a varying influence on the intensity of the flow-induced lift forces.

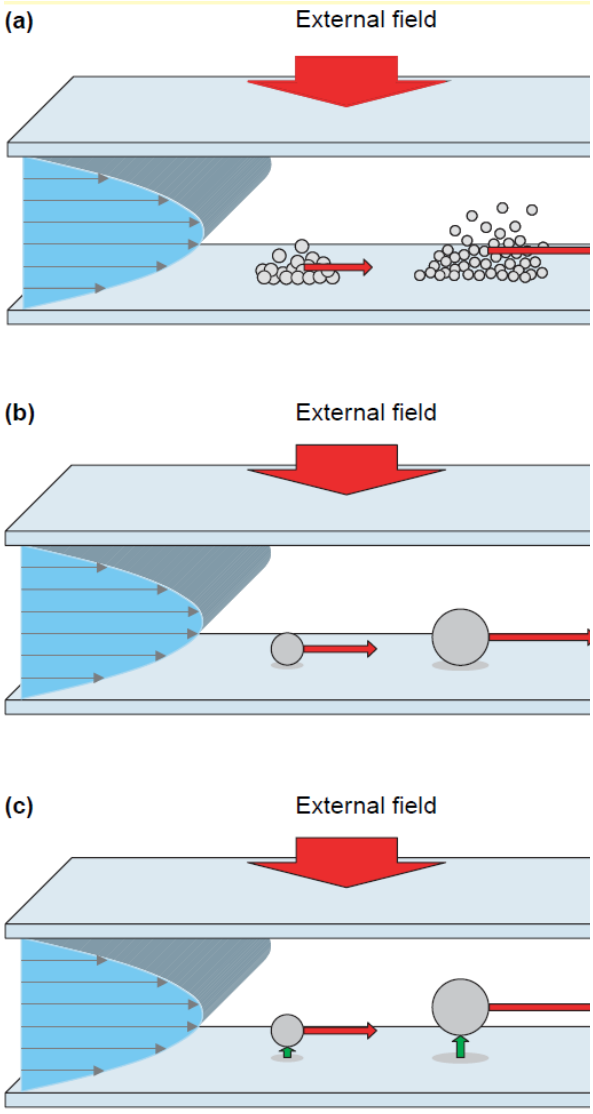


Figure 2.1. Most-frequently-used FFF operating modes. Different mechanisms of separation for particles of different size. (a) Normal, (b) steric and (c) hyperlayer mode.

Non-equilibrium, Earth gravity-assisted dynamic fractionation (NEEGA-DF)

GrFFF has been used to sort different human stem cells and to enrich leukapheresis samples from healthy human donors. [62]Due to its high simplicity and biocompatibility, GrFFF poses no technical issues for the fractionation of cells under easy sterilization conditions and the fractionation device, once used, may be disposed. However, application of GrFFF method to adherent cells requires cell sedimentation at the accumulation wall, which tends to cause cell adhesion to the wall and cell-cell aggregation/stacking. This can drastically reduce cell recovery, and also affects cell functionality after fractionation. We have developed a method based on a novel modification of the GrFFF process. The method is named Non-equilibrium, Earth gravity-assisted dynamic fractionation (NEEGA-DF). NEEGA-DF does not require cell sedimentation at the accumulation wall.[63] Compared to GrFFF, in NEEGA-DF cell contact and adhesion with the separation device are avoided by in-flow injection, by the absence of stop-flow cell sedimentation, and by using elution flow rate values able to generate hydrodynamic forces that are intense enough to lift and keep cells away from the channel wall. Since during separation cells are suspended in a fluidic condition, they acquire features that

[62] B. Roda, P. Reschiglian, F. Alviano, G. Lanzoni, G.P. Bagnara, F. Ricci, et al. (2009) *Journal of Chromatography A*, 1216(52), 9081-9087

[63] P. Reschiglian, B. Roda, A. Zattoni, G.P. Bagnara. Method and device to fractionate stem cell. Patent PCT/EP2007/054226.

may be different from their native, adherent state. The fractionation process is therefore based on the differences in cell features that are dynamically acquired during flow-assisted fractionation under the combined action of the flow stream, the gravitational field, and the hydrodynamic lift forces. The flow rate values applied guarantee low shear stress on cells. After fractionation is completed, cells can return to the adherent state, and the native physical features are fully restored. Consequently, during NEEGA-DF cells never come in contact with the separation device, and adherent stem cells can be thereby separated and collected without adhesion to the wall and cell-cell aggregation. This allows high cell recovery and full maintainance of cell viability and differentiation features.

We have applied NEEGA-DF to a protocol able to purify, distinguish and sort human mesenchymal stem cells (hMSCs) from various clinical specimens.[64] [65]hMSCs are adherent, multipotent stem cells that can be isolated from various connective tissues such as bone marrow, fetal membranes, adipose tissue and dental pulp. hMSCs are considered promising candidates for clinical applications based on cell-therapy approaches. This is also because, other than multipotency, they have the very unique characteristic of not provoking an immune response when administered into another individual. They are therefore sought as ideal source for stem-cell allogenic transplantations. They exhibit differing lineage-

[64] B. Roda, P. Reschiglian, A. Zattoni, F. Alviano, G. Lanzoni, R. Costa, et al. (2009) A tag-less method of sorting stem cells from clinical specimens and separating mesenchymal from epithelial progenitor cells. Cytometry Part B (Clinical Cytometry),76B, 285–290.

[65] B.Roda, G. Lanzoni, F. Alviano; A. Zattoni, R. Costa, A. Di Carlo, C. Marchionni, M. Franchina, et al. (2009). A Novel Stem Cell Tag-Less Sorting Method. Stem Cell Reviews and Reports, 5, 420–427

commitment yields and differing expression levels of pluripotency markers, very likely because of the presence of dissimilar progenitor cells. This makes difficult to apply techniques based on immunotagging for hMSC sorting. For these reasons, they have been ideal case samples for the application of the NEEGA-DF protocol. The protocol can be applied to separating hMSCs from potential phenotypically different contaminants when cells are isolated from clinical specimens, thus allowing one to reduce the number of cell culture passages for MSC selection; to distinguish MSCs derived from different sources, and finally to sort stem cells from an MSC population isolated from a single source, obtaining the highest differentiation yield. The protocol then represents a new tool for tag-less stem cell purification and sorting of stem cells which can be easily integrated in conventional cell-sorting platforms to reduce time and improve fully functional stem cell yield.

Fractionation

System setup

The fractionation device was a ribbon-like capillary channel comprised of two polyvinylchloride walls sandwiching a thin foil of polyethylene terephthalate from which the channel volume had been removed. Channel dimensions were 2.0 cm in breadth, 0.025 cm in thickness and 30 cm in length. The ensemble was

sandwiched together using proper clamping systems, which may if necessary be removable clamping systems such as nuts, bolts or rivets (Figure 2.3a).

The fractionation device should be prepared with the following instrumental set-up, as shown in Figure 2.3b:

-a peristaltic pump, used to impart the mobile phase into the system, was connected at the channel inlet by means of a T-valve;

- the T-valve was connected to a PEEK inlet tube (L= 7 cm, i.d. = 0.750 mm, o.d. = 1/16”) screwed at the beginning of the channel wall used to allow flow and sample injection.

- at the fractionation device outlet, a UV/Vis detection system was connected to monitor the elution process, recording a signal at 600 nm;

- a fraction collector was connected downstream of the detector outlet to collect eluted cells.

-The overall system was placed in a laminar-flow hood to assess sterile conditions. The system was placed in a horizontal position to make the gravitational field act perpendicularly to the carrier liquid flow.

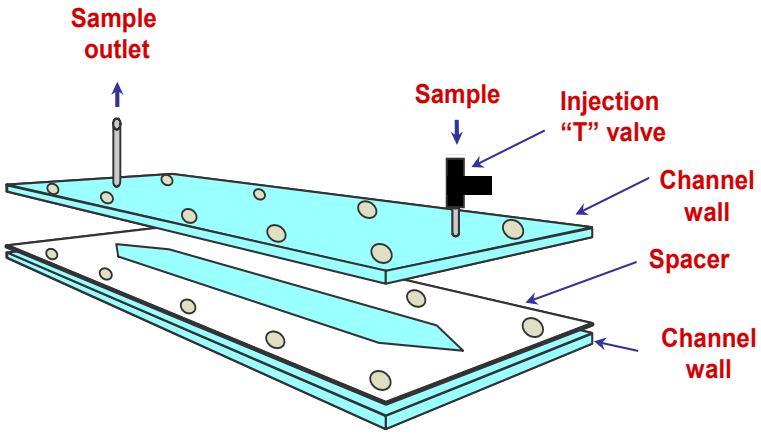


Figure 2.3a: Exploded view of the prototyped fractionation device

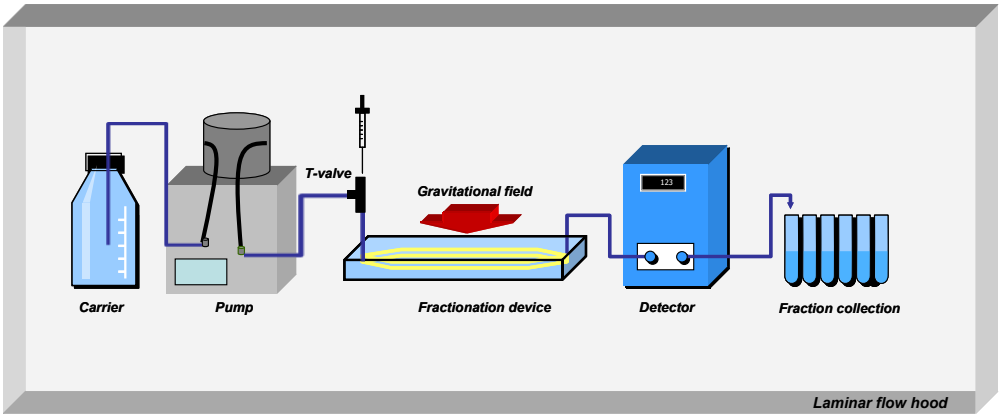


Figure 2.3b: Scheme of the prototyped fractionation system

Protocol

A representative protocol is schematized in Figure 2.4. It consists of:

1. Sterilization of the fractionation system and conditioning to be performed at the beginning of each working day:

-fill the fractionation system with the sterilization solution for 1 hour at 1 ml/min;

-fill the fractionation system with sterile water for 1 hour at 2 ml/min to thoroughly wash the system and eliminate active chlorine traces;

- fill the fractionation system with sterile mobile phase for 30 minutes at 0.5 ml/min before sample injection for channel wall conditioning.

- The 100-µL HPLC syringe to be used for sample loading is itself sterilized with the same hypochlorite solution and then washed twice with sterile water and finally with sterile mobile phase.

2. Preparation of a cell sample: cells are counted and resuspended in the mobile phase at a concentration of 3x105 cells/ml. Cells need to be properly maintained in suspension to avoid cell aggregation.

3. Injection of cell sample: a volume of 50 µL of the suspension are injected into the channel PEEK inlet tube (L= 7 cm, i.d. = 0.750 mm, o.d. = 1/16”) by means of an HPLC syringe. The flow is stopped for some seconds to allow all samples to enter the channel; then by means of a T-valve the inlet port is closed.

4. Elution of cell sample: after injection, the flow is immediately restarted and set at 0.46 ml/min. After a relatively short period of time from injection (about 30 minutes), cell elution was complete.

5. Cell fraction collection. When necessary, eluted cells are collected at the fractionation device outlet as selected fractions.

6. Isolation of fractionated cells, and possibly further characterization/selection and/or in vitro expansion thereof.

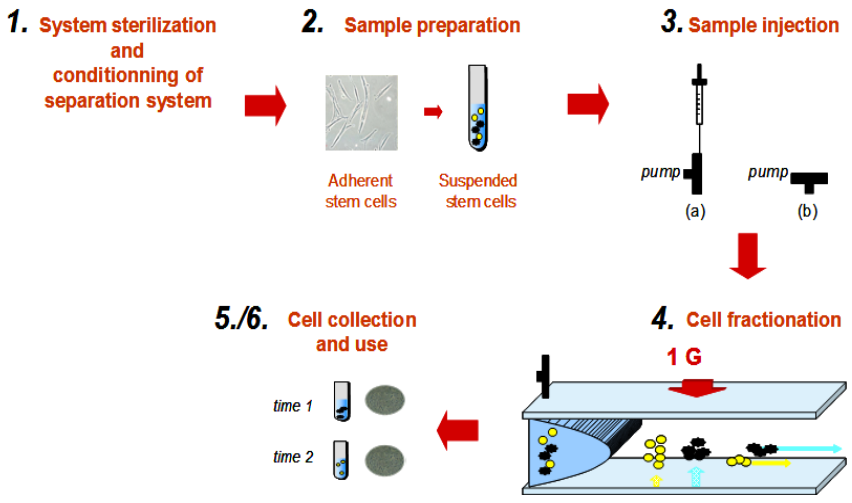


Figure 2.4: Stem cell fractionation protocol

Moving from the NEEGA-DF technique, the whole technology was developed and transformed in a brand new instrumentation with the aim of bring the new tool on the market of regenerative medicine.

The property of the invention : Stem Sel srl

Stem Sel® Srl is a spinoff company participated by AlmaCube Srl, the incubator of the University of Bologna and Unindustria. The business project is also supported by Regional actions to support the creation, development and improvement of the business idea. It is based on the development, the engineering, the manufacturing, and the commercialization of the instrument Celector® (and related disposables), currently at prototype phase. This product is based on an innovative and patented technology for the separation and selection of human stem cells from adult tissues, such as “discarded” tissue (e.g. placenta, umbilical cord blood or lipoaspirate). The aim is the characterization, the quality control and the future use for cell therapy applications in Regenerative Medicine.

Stem Sel® thanks to this team made by strong multidisciplinary characters and highly qualified scientists, has the perfect combination of professional profiles to guarantee the prospect of success.

The Product: Celector®

Celector® is the novel technology for cell separation that has the key advantage to sort both cells from rough tissues rough and ex vivo cultured cells without any sort of manipulation. This allows cells maintaining their native proprieties, and stem cells their potential. No immuno-tagging is required for cell sorting, and the absence of any type of cell manipulation allows passing regulatory restrictions. Moreover, our tag-less technology allows for selection/sorting of those stem cell

types for which there are not, as yet, efficient technologies on the market. In fact, fluorescence/magnetic-activated cell sorting (FACS/MACS) technologies do manipulate cells using immunomarkers, which otherwise might be not available or be poorly specific to efficiently select highly potent stem cells such as the mesenchymal stem cells (MSCs). Nevertheless, MSCs are among most-promising adult stem cells for clinical applications. Novelty and unique features of Celector® then make it a potential “leader tool” among technologies and devices for cell therapies.

Stem cells are distributed in all tissues. They can be then sorted from such sources. However, their localization in each source tissue is not well defined, and they cannot be identified in a specific district isolated from all different cells, which are more differentiated and originated from the stem cells. Moreover, the lack of homogeneity in pluri/multipotent SCs severely hinders a definition and standardization for successful stem cell-based therapies. Finally, cell-type-specific markers such as cell surface proteins are limited known, and they often recognize multiple members of a SC lineage. Stem cell recovery and functionality are also affected by immunolabeling. Methods that are less dependent on the identification of particular markers for SC subpopulations, and which exploit differences in biophysical cell characteristics, are therefore promising when it comes to identify and sort homogeneous SC subpopulations MSCs are am0ng most-promising adult stem cells for cell therapy applications.

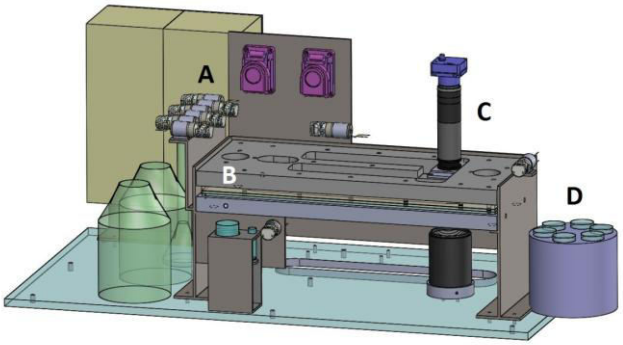
They are adherent, multipotent SCs that are present in quite a few “discarded” tissues. However, MSCs express a so-rich and diversified panel of surface

antigens that limits the possibility to efficiently “distinguish” MSCs on a immuno-phenotypical basis. To obtain homogeneous stem cells, particularly MSCs, which are properly characterized, safely usable, and in sufficient number, proper methods of isolation/enrichment/sorting are then required.

Celector® does operate sorting with no immuno-tagging. Its proprietary separation process exploits differences in the intrinsic characteristics of the cells, which include size, density and surface properties. Cell sorting occurs in a biocompatible fluid (PBS-phosphate buffer saline, physiological solutions, culture media) through the sterile fluidic device that can be disposed once used. The separation process avoid cell contact, and consequent adhesion on the separation device, and cell-cell aggregation by using in-flow injection of cells and a proprietary combination of different flow stream rates able to keep cells away from the channel walls, and be swept down the separation device at different velocities. This can make different cells be collected at different times in sundry containers.

The flow rate values typically applied guarantee low shear stress on cells. After fractionation is completed, native physical features then are fully restored. This allows high cell recovery and full maintainance of cell viability and differentiation features.

The Celector® is composed by this subsystems listed below and described following.



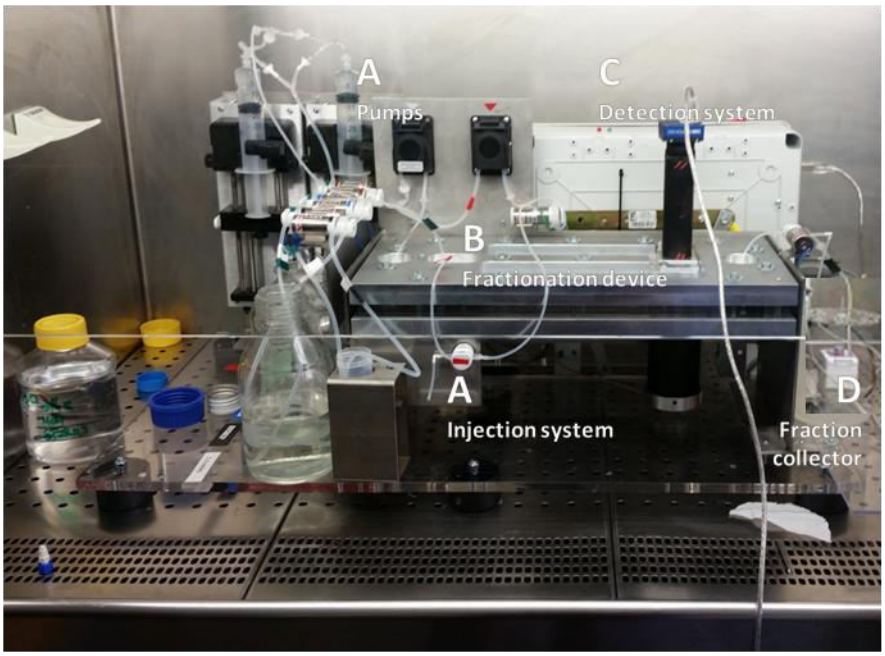


Figure 2.5: overview of Celector®

A. INJECTION SYSTEM

1. Autosampler

2. Pump (PC controlled) and valves

3. Biocompatible fluids for cells separation

B. FRACTIONATION DEVICE

4. Capillaries for fluidic transport of cells to the separation device and to the detection system

5. Proprietary cell fractionation device using the proprietary process for cell separation (single or multi-channel option)

C. DETECTION SYSTEM

6 Optical detection (PC controlled): it counts, record and recognizes all kind of fractionated cells

D. FRACTION COLLECTOR

7. Fractionated cell collector (PC controlled)

Hardware and functions

Subsystems of Celector

The instrument is composed of several subsystems dedicated to the carrying out of different operations that characterize the operational mode.

In Figure 2.1 are represented the different subsystems of the instrument, described in the following paragraphs.

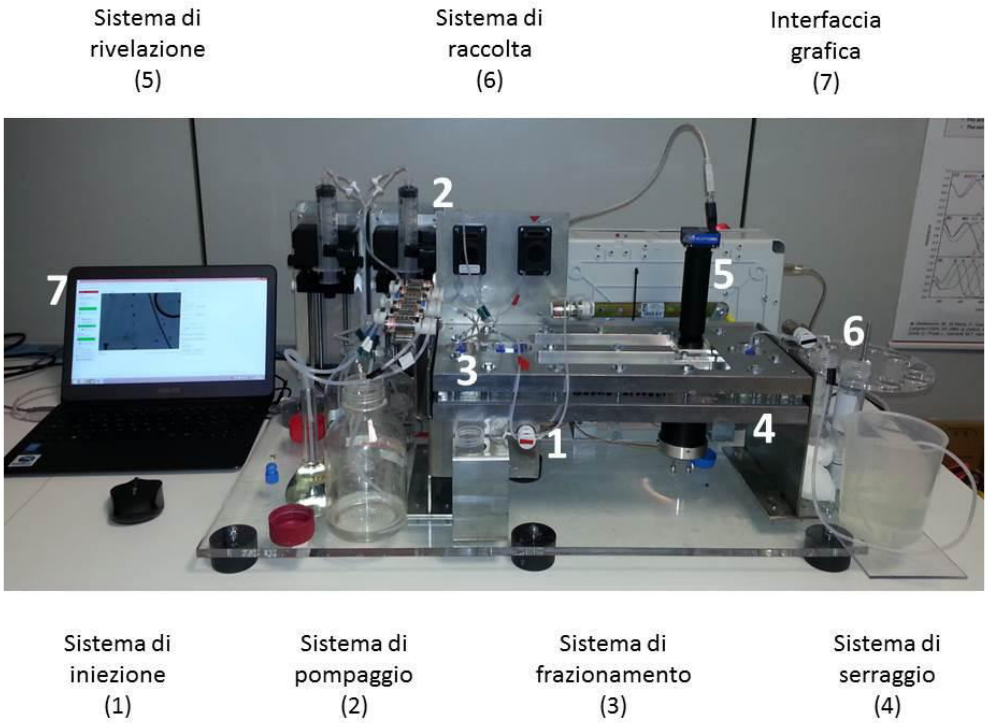


Figure 2.6: subsystems composing Celector®

Injection system / Autosampler

The injection system allows inserting the cell sample into the fractionation system, sucking the sample from the tube and injecting it automatically into the fractionation system, according to previously defined protocols and setting up defined by the operator. The injection procedure is performed by a dedicated peristaltic pump and a series of valves, which controlled via software drive the sample from the Eppendorf to the fractionator by silicon tubes.

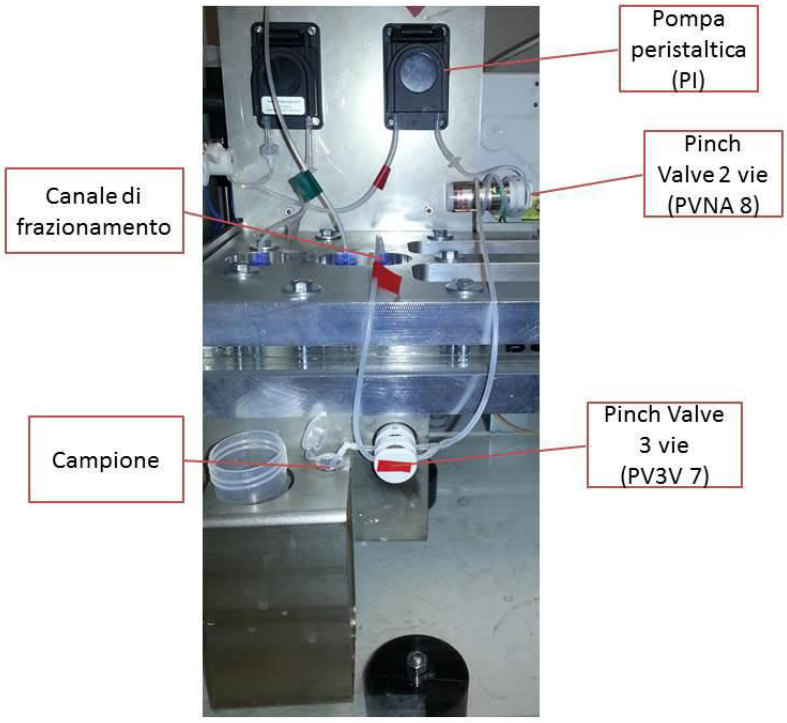


Figure 1.7Injection system

Fractionation system

The fractionation system is the heart of the technology, which perform the separation of cells as discussed before. It is part of consumable kits because of giving the possibility to replace the fractionator when desired to avoid contamination sample-to-sample or run-to-run. It consists of a multilayer disposable device of plastic material where both the separation flow and the cell suspension are injected through an automated system of capillary tubes. Figure 2.8 represents the fractionation channel consisting in 5 layers of PMMA, laser cutted to create the fluidic circuit. The layers are sealed to each other by means of a clamping system.

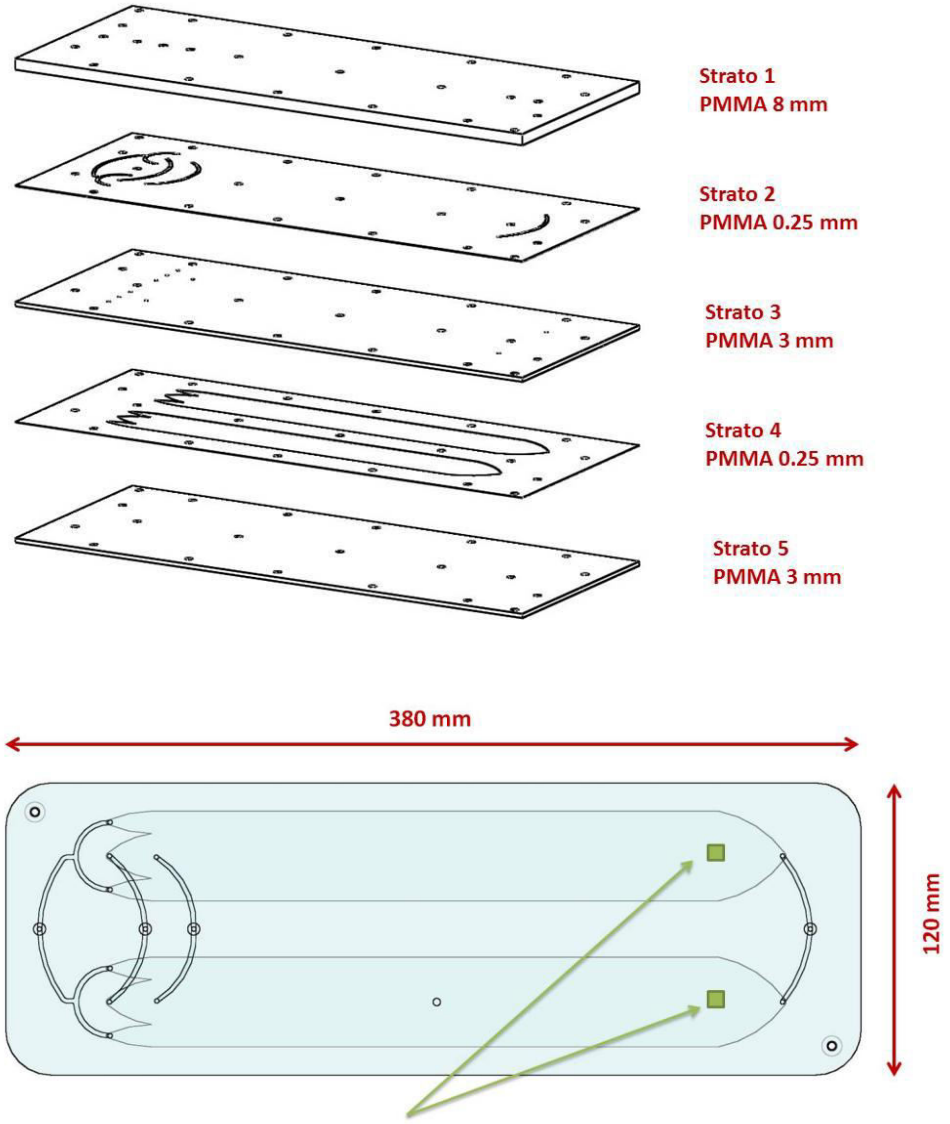


Figure 2.8 layers composing the channel in exploded view (above) and top view (bottom)

Clamping system

The clamping system is essential to seal the fractionation system ensuring the hydraulic seal of the transport fluid and the cell sample. In figure 2.9 it is

represented the system employed in the prototype version, and consists in two 20 mm thick aluminum plates held together by bolts tightened at a specific force.

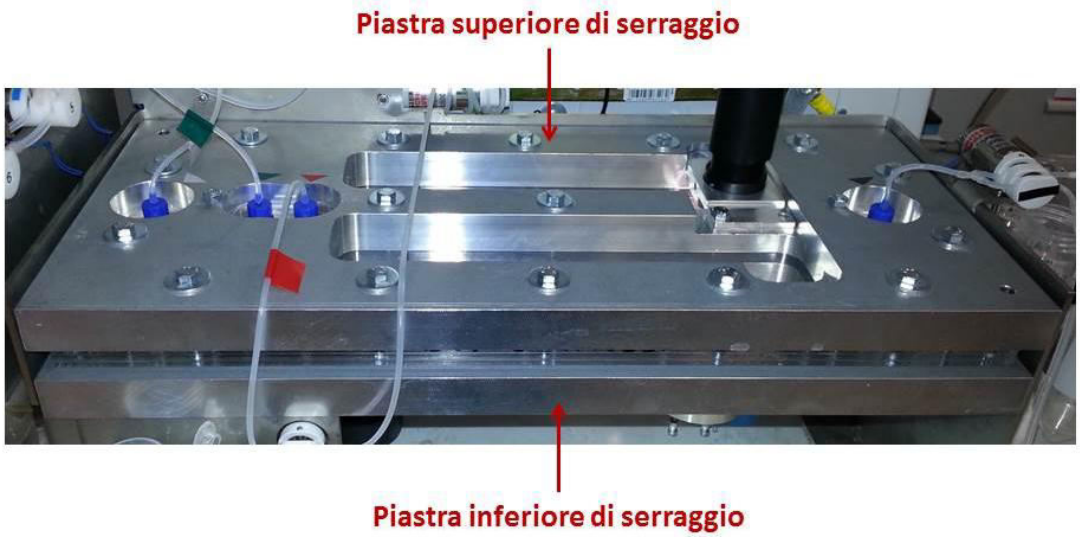


Figure 2.9 clamping system consisting in Aluminum plates. The most reliable clamping system at now.

Pumping System

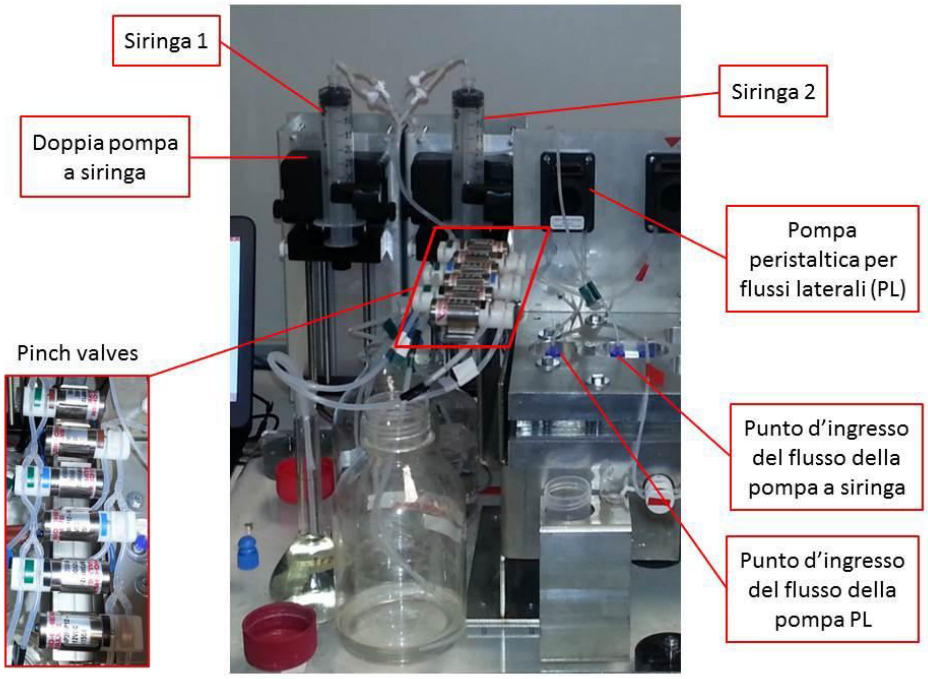


Figure 2.10 pumping system: double syringe pump and stepper motor peristaltic pumps

The pumping system (fig. 2.10) manages all the flows, is PC controlled and set up to create the appropriate flow profile inside the separation device. Pumps are connected each other and to the fractionation system by the piping system. The syringe pump was implemented for the generation of the central flow, which is responsible of the sample separation, in reason of the continuous and not-pulsed flow able to produce. Conversely the peristaltic pumps show a pulsed flow, but were selected for the lateral flow dispensing and for the injection procedure because the irregular flow is not important for their functions. The first one rotate so slowly (at a flow rate in the order of 1/10 respect to the central flow) that the frequency of pulsation is not appreciable. The second one operates out of the

separation chamber except during the in flow injection for small defined interval of time, but it's convenient using a stepper motor pump in reason of the accuracy of the volume processed, leading to a repeatable and standard volumes handling.

Detection System

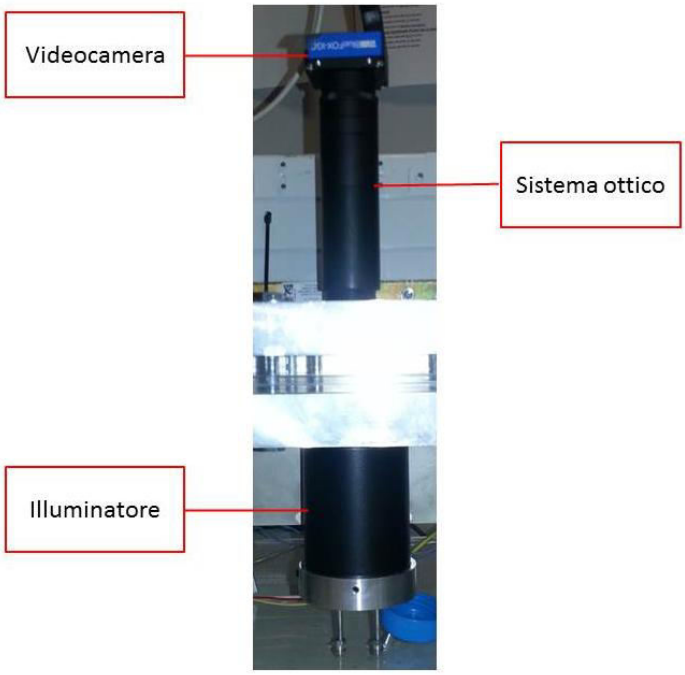


Figure 2.11 Detection system

The optical detection system (fig. 2.11) is used for the on-line counting of the processed cells during the separation. A dedicated software was developed by the internal team in order to control the separation process, returning the cell amount for each fractions, characterize the morphology of different populations, manage the collector system during the fraction collection.

The main hardware components of this system are a ultrabright LED illuminator, a semi-plan objective 4X magnification and a BlueFox MatrixVision camera.

Collection system

The collection system (Fig. 2.12), allows the automatic collection of the cell selected fractions exit from the fractionation system in Falcon tubes both of 15 ml and 50 ml volumes, according to user-selected collection times and preferred tubes. An holed PMMA circle rotates thanks to a stepper motor, shifting from a tubes to the other.

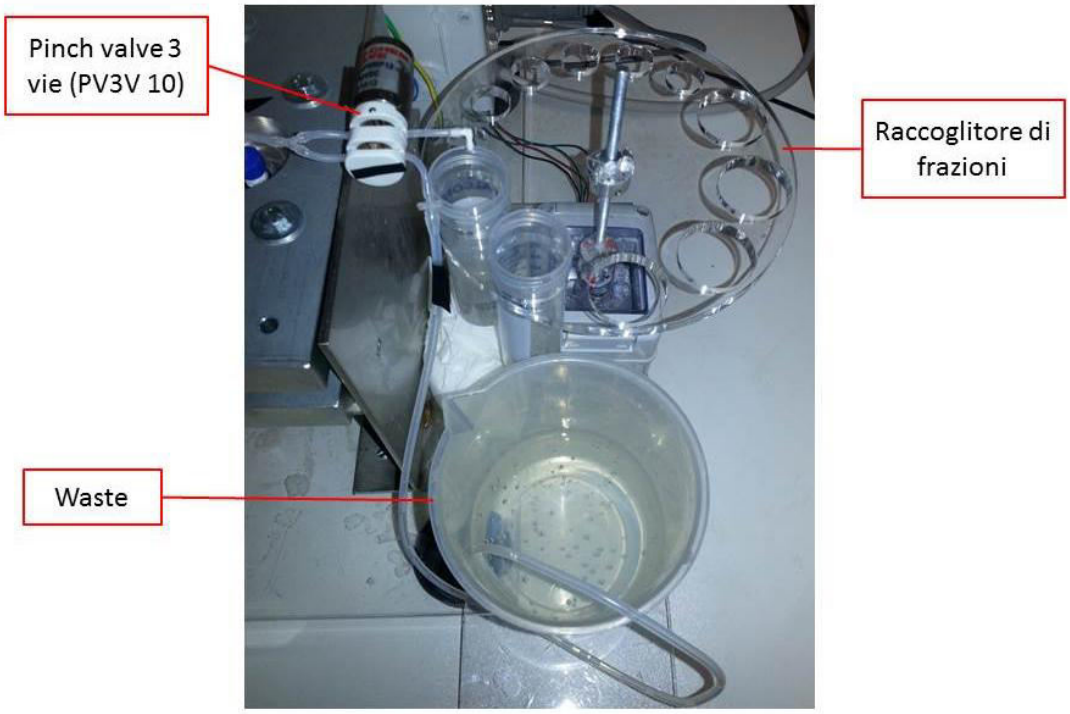


Figure 2.12 collection system

Graphic User interface (GUI)

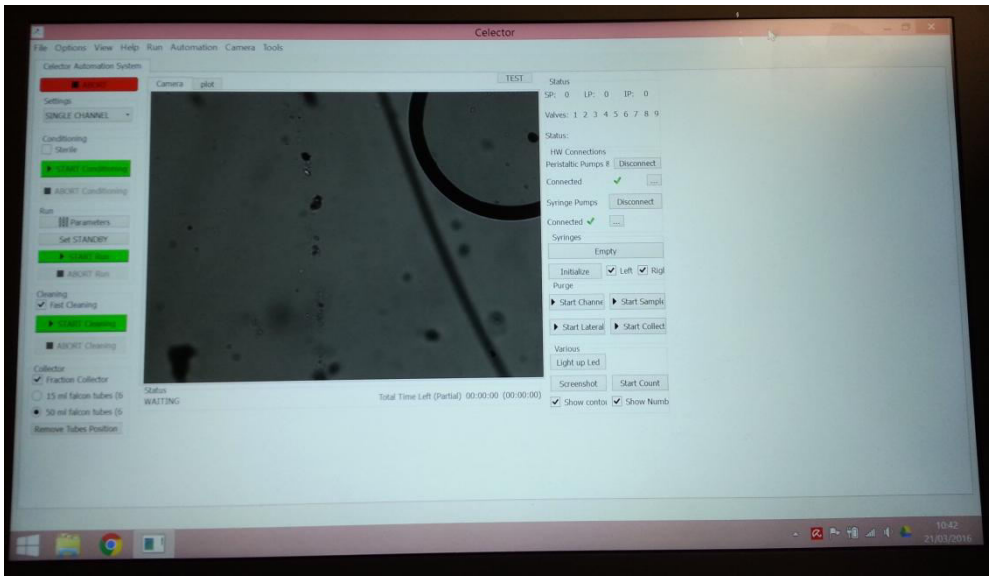


Figure 2.13 GUI

The Celector® prototype is controlled via a graphical user interface illustrated in Figure 2.13 can be installed on every PC. The graphical interface make the instrument easy-to-use also by not specialized personnel, thanks to the possibility for users to interact with electronic devices through graphical icons and visual indicators.

External case

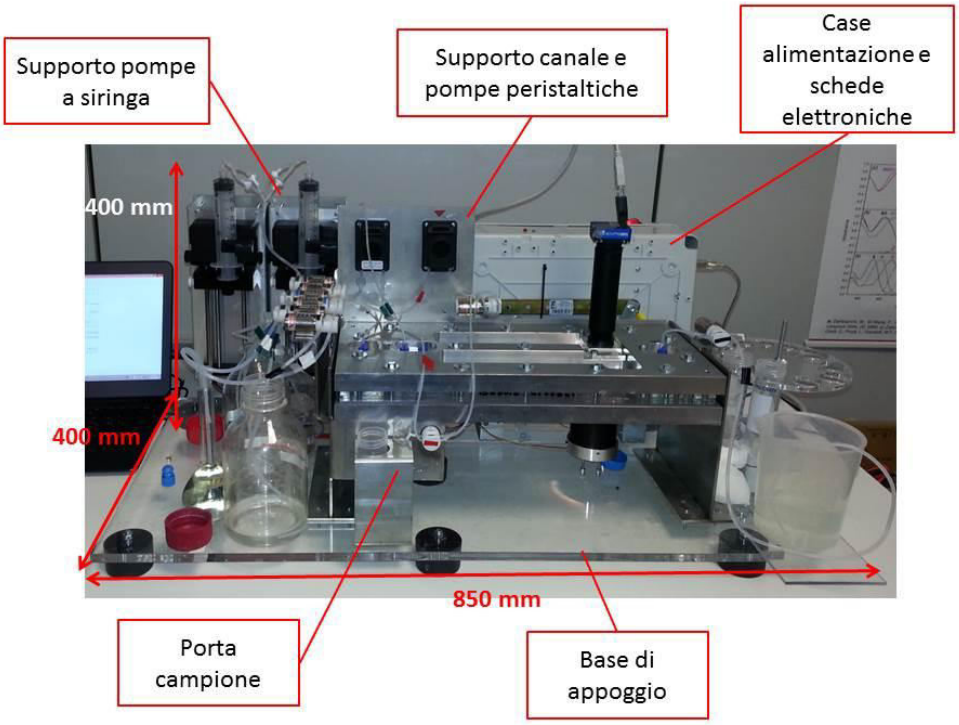


Figure 2.14 Mechanical support

The mechanical structure with frame function of the prototype is shown in Figure 2.14. The size of the current prototype is about 850 x 400 x 400 mm. Despite the inappropriate dimension for a Medical Device or a Biotech Lab Tool, the prototype fits in the laminar hood so that cells can be prepared and separated in sterile conditions, feature that allows also long term biotech characterization of the separation product without any contamination. The development of the external case will permit maybe also to move the instrument from a hood to another or to process the separation out of the hood maintaining the sterility inside the case.

Piping system

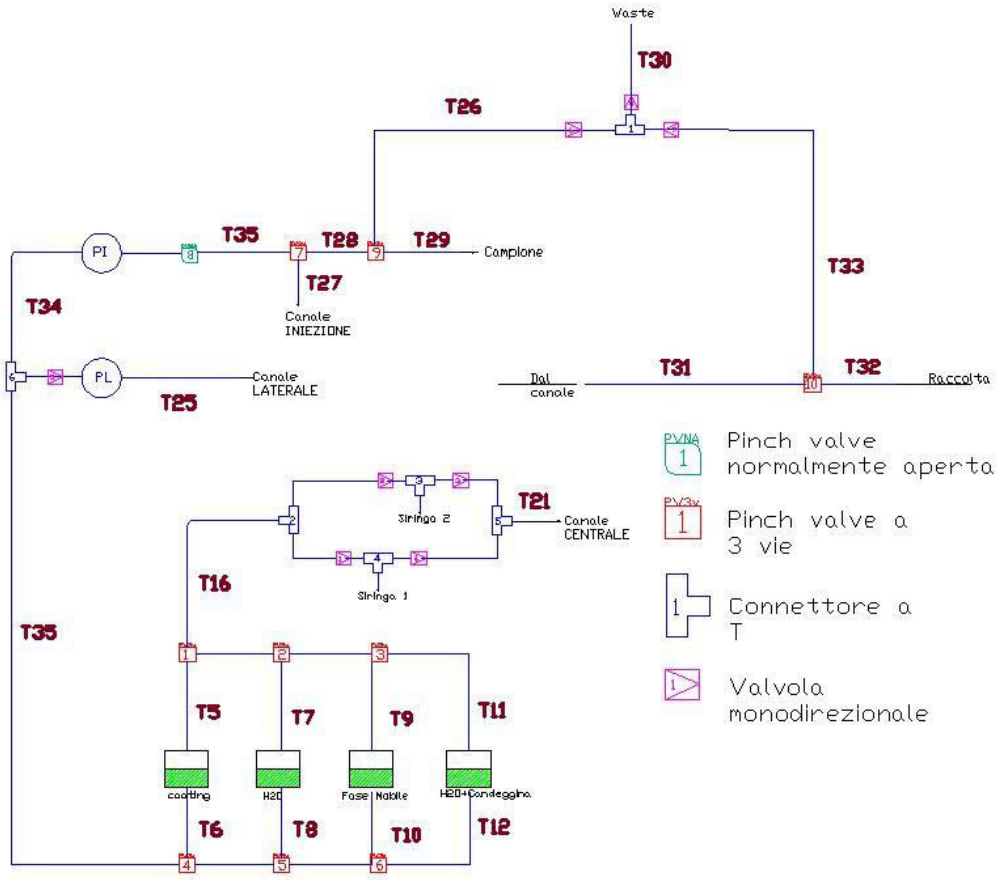


Figure 2.15: hydraulic circuit

The hydraulic system is thought to be disposable and replaceable when needed. Moreover was developed in compliance with the requirements of Medical Device class IIb, so materials and methods were developed and tested in this point of view. These considerations lead to a circuit that has not to be touched from components of the machine, so it is composed by silicone tubes, connected through adaptors, valves and connections MD compliant and assembled in clean room (at the prototype stage is only sterilized once assembled). The flow in silicone tubes is managed by electroclamp valves which open and close tubes clamping

them by a solenoid, so these valves act on the external surface of the tubes never enter in contact with.

Operational mode: the main phases

1. Purge

Once switch on the instrument start the purging phase in order to eliminate air contained in piping and in the fractionator system. Subsequently, the tubes are filled with sterile solution (demineralized water or PBS).

2. Sterilization

During this phase are eluted in the fractionation device in succession sodium hypochlorite and double-distilled sterile water. This is necessary to further sterilize the device in the case has already been used and washed. It can be omitted in case the new consumable has just been inserted and then a phase of sterilization is not required.

3. Conditioning

During this phase in the fractionating device are eluted in succession the coating solution (PBS and 1% BSA) , in order to saturate plastic free sites that would bond

to cell surface holding it on the accumulation wall, and the the mobile phase solution (usually PBS and 0.1% BSA) to wet very well the channel for optimal separation conditions.

4. Running

Represents the actual procedure of separation in which the cell suspension is injectied to be separated and then collected for next proposal. Eluted cell fractions are automatically collected in the appropriate tubes.

5. Cleaning

This phase provides for the elution in succession of sodium hypochlorite and sterile distilled water inside the fractionation device in order to clean the hydraulic circuit from cell residues and after rinsing the circuit avoiding the deposition of salts.

Software interface Operations

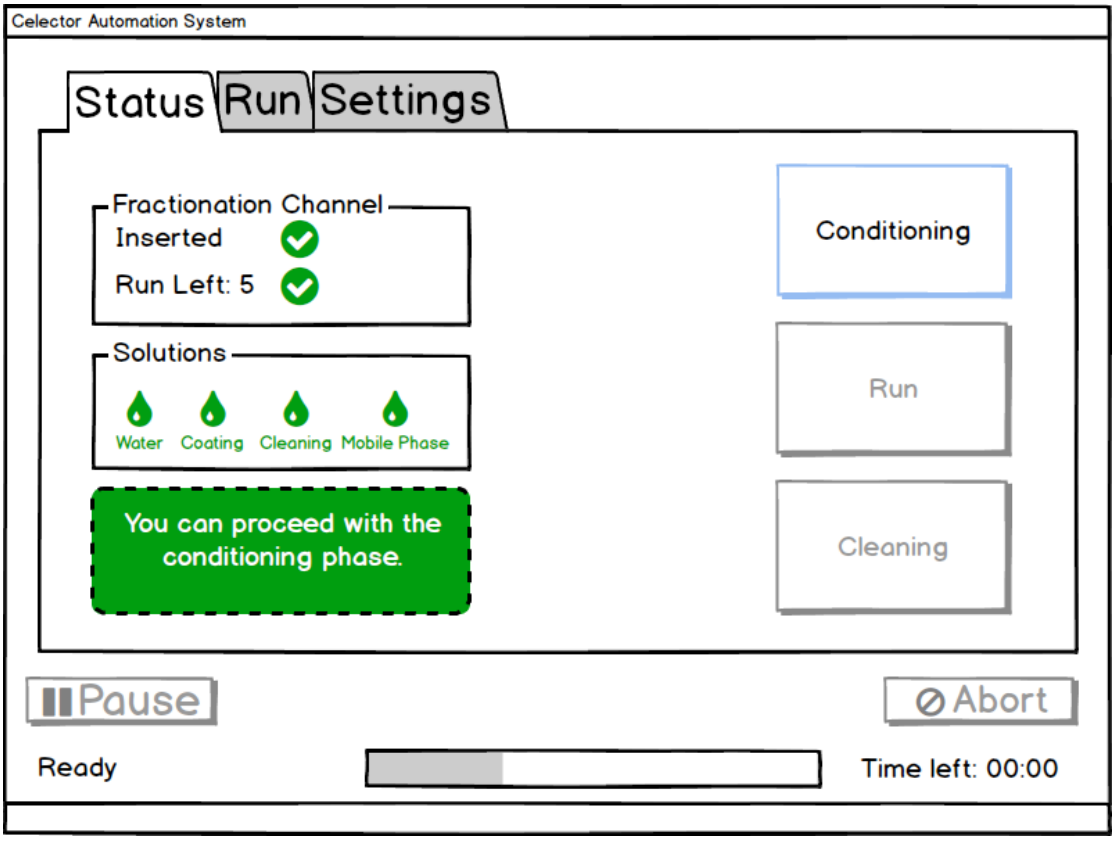
During the instrumental setting and parameters definition, to maximize the result of the separation and cell count, the researcher will have to consider that:

- Celector can separate at each run from 50,000 to 1.000.0000 of cells in a volume between 50 and 200 μl. A run has an average duration of 20

minutes and it is possible to operate more consecutive run. Summarizing it is possible to separate from 150,000 to 3,000,000 cells per hour;

- The central flow is at the base of the separation, so it has to be choose evaluating the dimension and shape of cells. Default flow rate is set at 1 ml/min

1) Presence of the consumables and separation liquids



Figures 2.16: Ready Status Channel

Once switch on the Celector and played the software, the latter this will control the fractionation channel presence inside the machine and will give an output to the user.

- If the fractionation channel is already present, the user can proceed to the next operation. The channel can be used for a limited number of runs. When the limit is exceeded the channel will have to be replaced because it can no longer perform good quality separations.
- If the fractionation channel is not present the software will ask the user to place the consumable before continue

Once the channel is inserted the user must specify whether it is a single channel or double channel to set up the fluidic regime.

Tubes

Tubes are parts of the disposable and have to be set up in the instrument. A colored tubing set help the user in the placement. It was programmed to create a video reproducing the set up of piping.

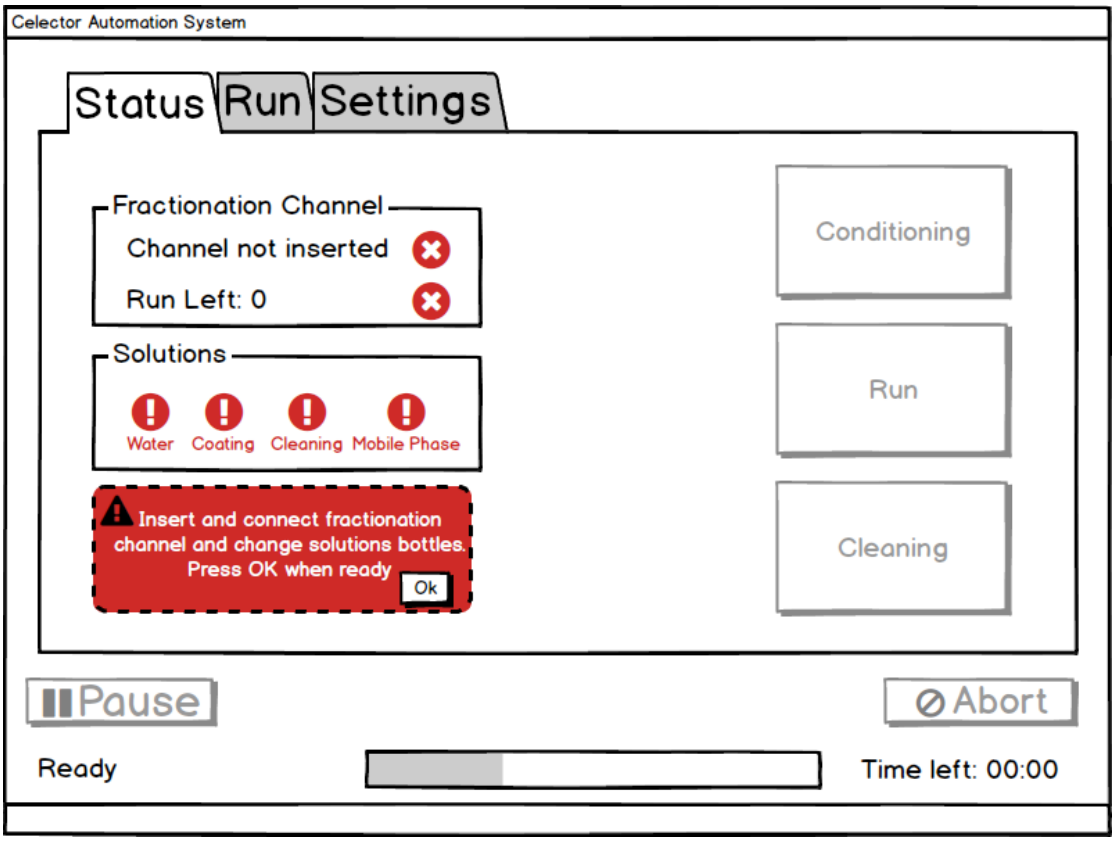
Containers and liquids for separation

The user must ensure that you have enough mobile liquids for all the phases, and placed in the right order. Stem Sel will supply the liquids for the different operational phases:

- Bidistilled water
- Coating solution: phosphate buffer saline (PBS) and Bovine Serum Albumine 1%
- Running solution: phosphate buffer saline (PBS) and Bovine Serum Albumine 0.1%
- Cleaning solution: sodium hypoclorite 30%

All the solution will be sterilized and filtred. It’s under evaluation the typology of the liquid containers.

If liquids are not sufficient for the next phase, the software stops the instrumentation.



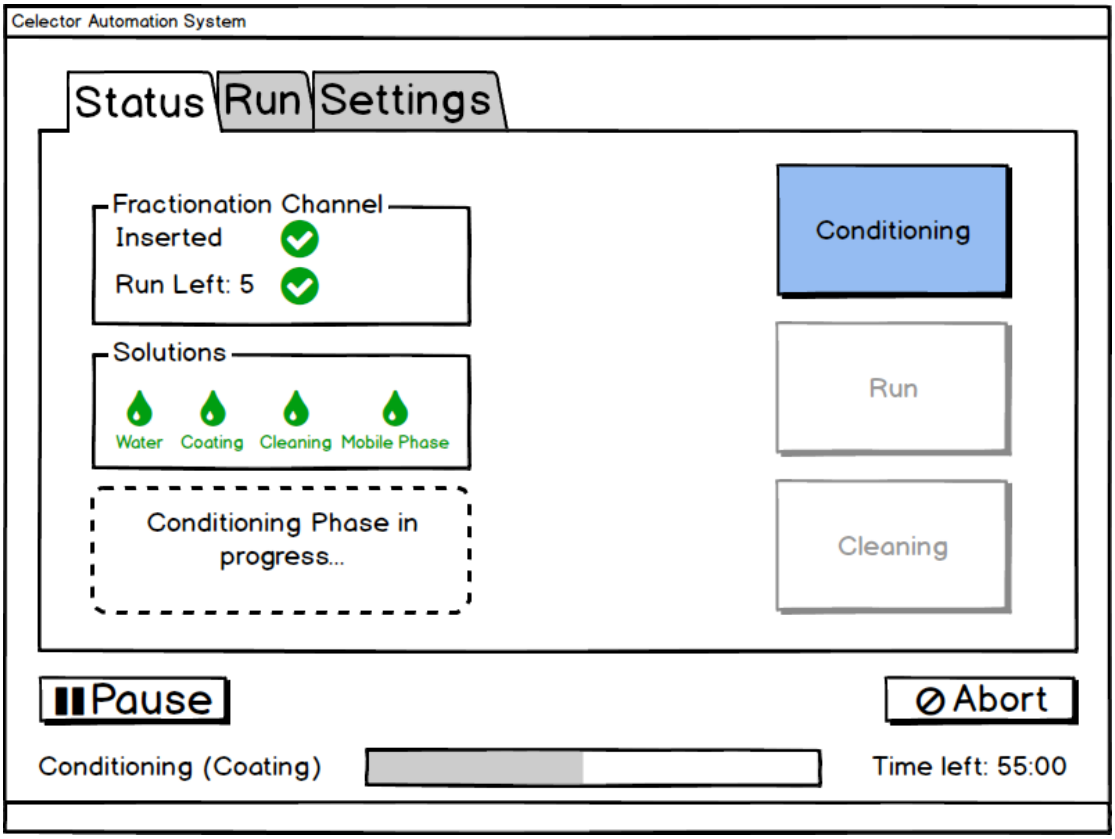
Figures 1.17: Not Ready Status

2) Conditioning

The conditioning phase consists in the coating phase and wetting of the fractionator.

The SW allows to decide if conditioning have to be performed in sterile conditions or not. In the first option the cleaning solution is pumped through the circuit and then rinsed by water.

At each stage of operation in the lower part of the screen it will be indicated the status of the instrument from a scroll bar, the missing time to start separations and pause buttons and abort.

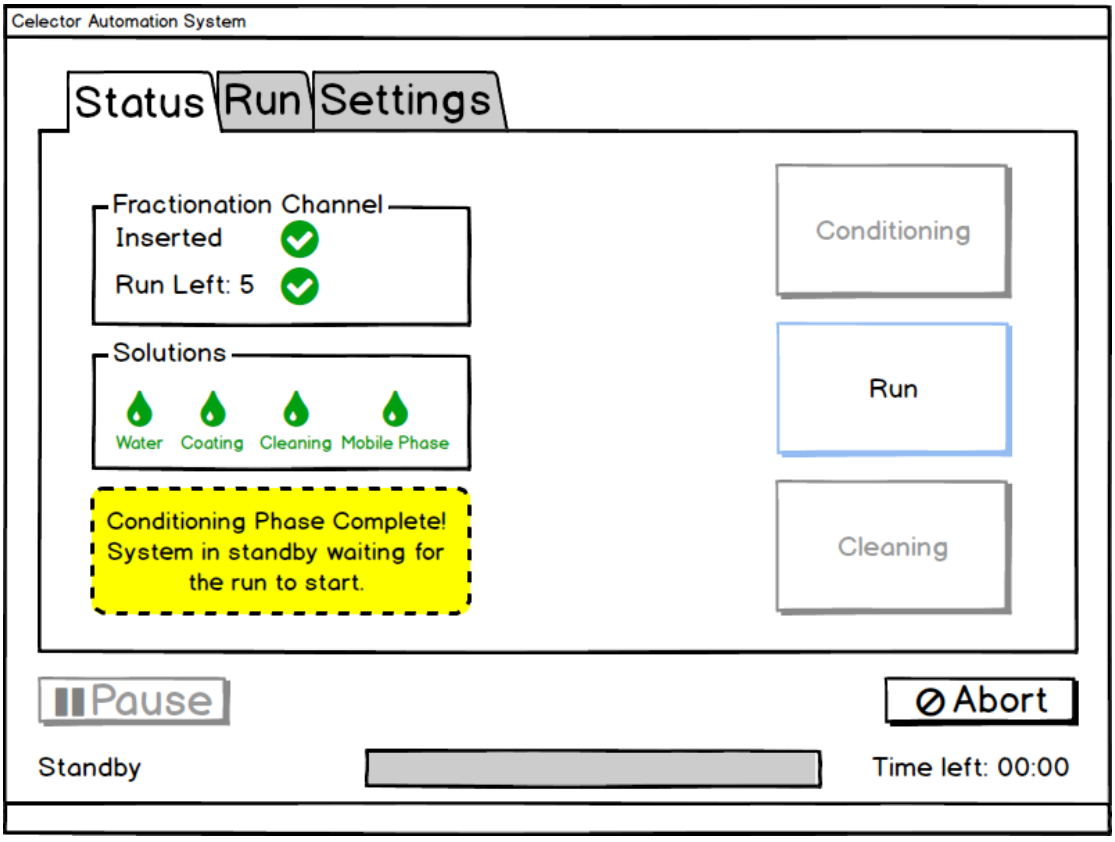


Figures 2.18: conditioning

3) Standby

In this stage, the machine sets itself in the Standby mode to save liquids (central and lateral flow slower)

The phase of Standby also active at the end of each run automatically, so as to allow the operator to perform different jobs ensuring minimal waste of fluids and preserving the collected fractions as set by the operator.



3b) Preparation of the sample

For the first run you will have to prepare the sample as specified in the manual under the following guidelines:

- the cell suspension should be prepared in eppendorf 1.5 or 2 ml

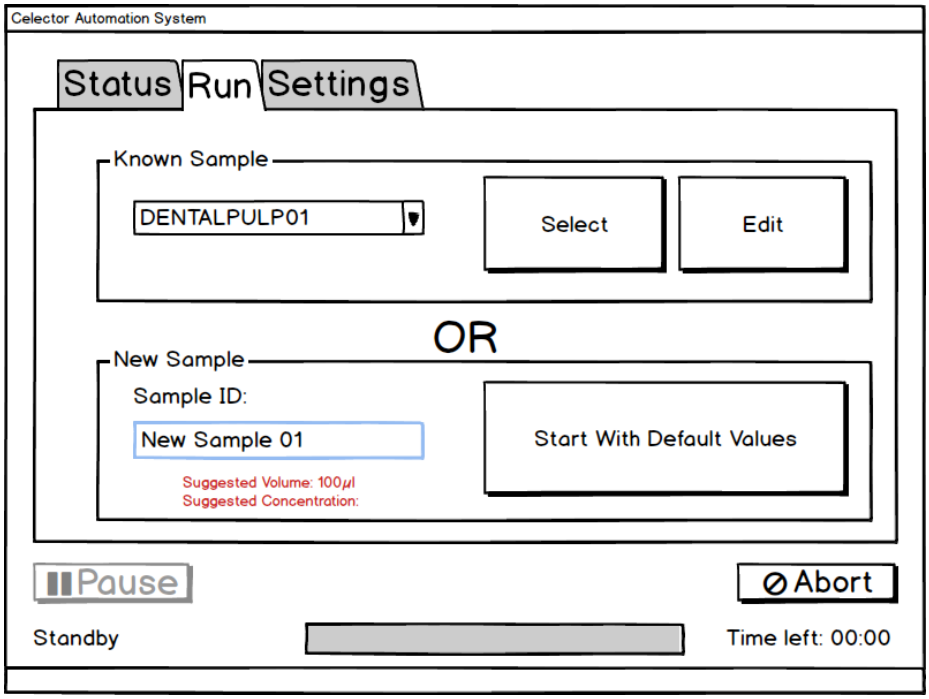
- cell concentration is around / $3x10^6$ ml diluted in PBS (phosphate buffered saline)
- $6x10^5$ cells are prepared and bring to volume with PBS at 2 ml

Celector processes about 100 μl/run and is able to process automatically, through a sequence of run, the totality of the preparation volume.

4) **Running**

At the beginning the user decides if the sample is “known” or “unknown”, so if the parameters to insert have been previously saved.

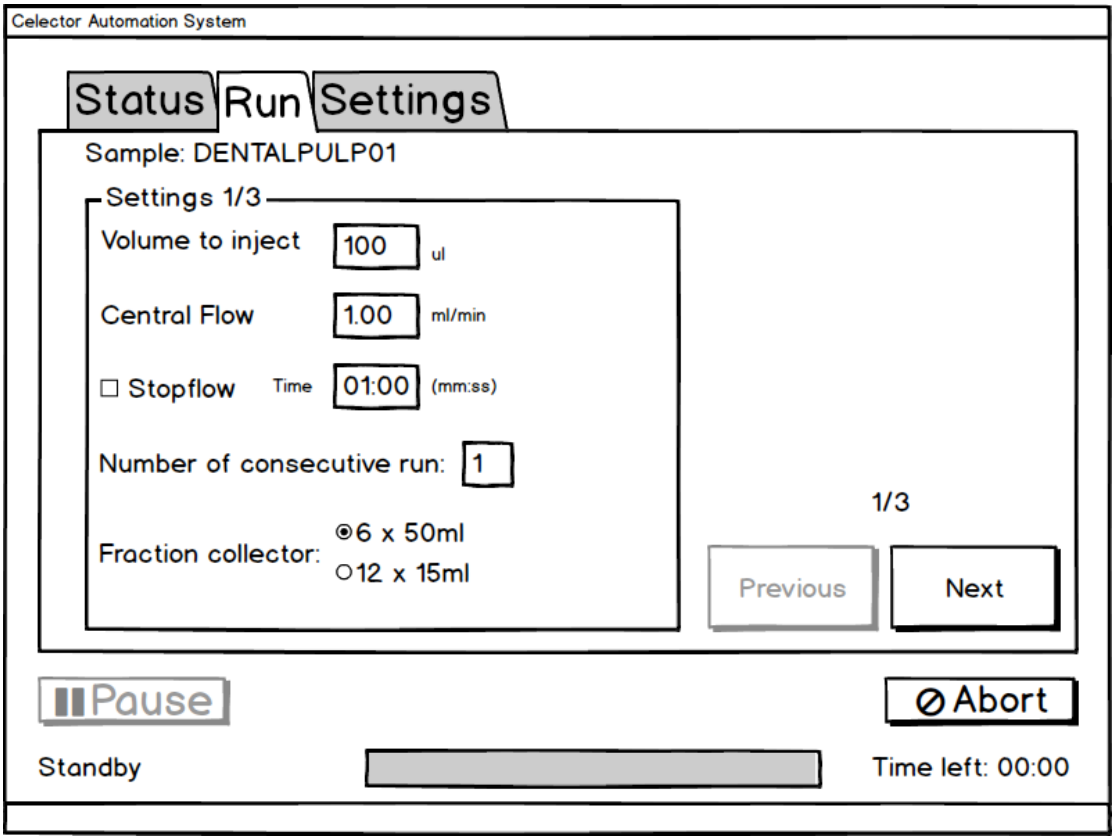
In the first case the parameters allows the counting from the first run, in the second case have to be performed a series of run to define the paramenters to enter.



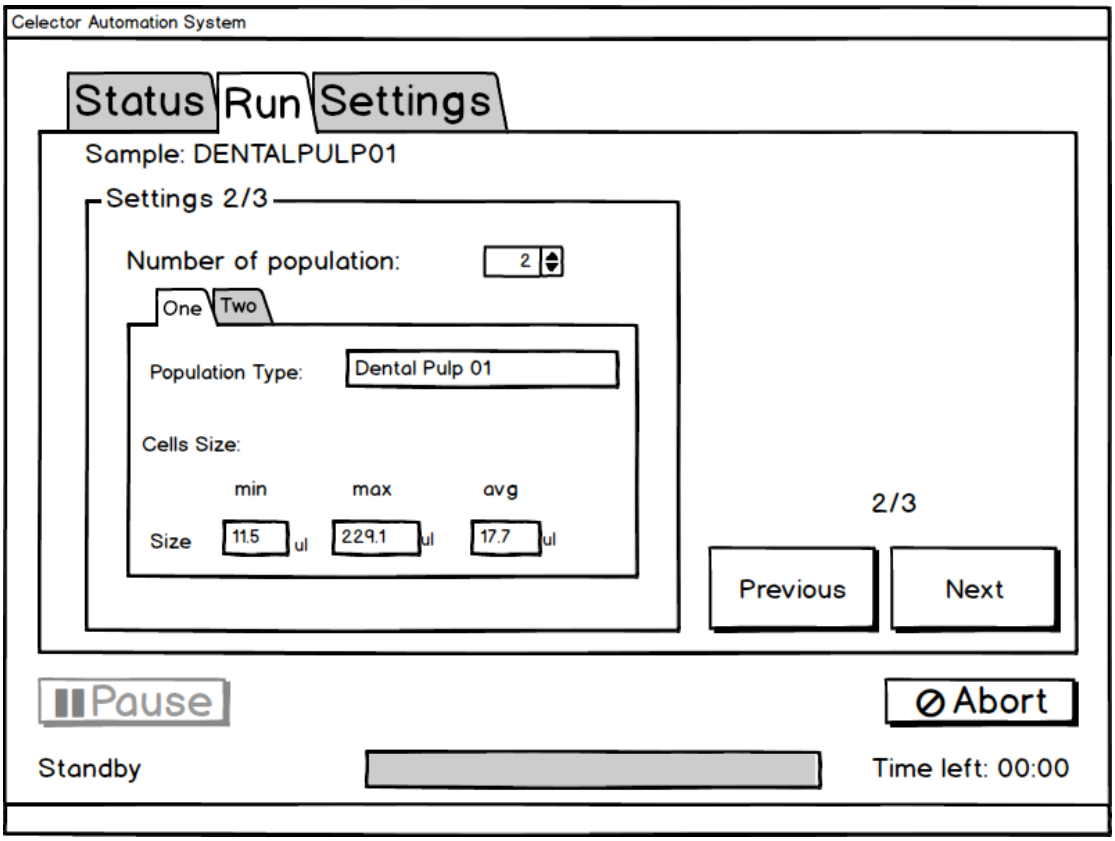
Figures 2.19: Choosing the run not yet analyzed sample

The parameters that can changed or evaluated are:

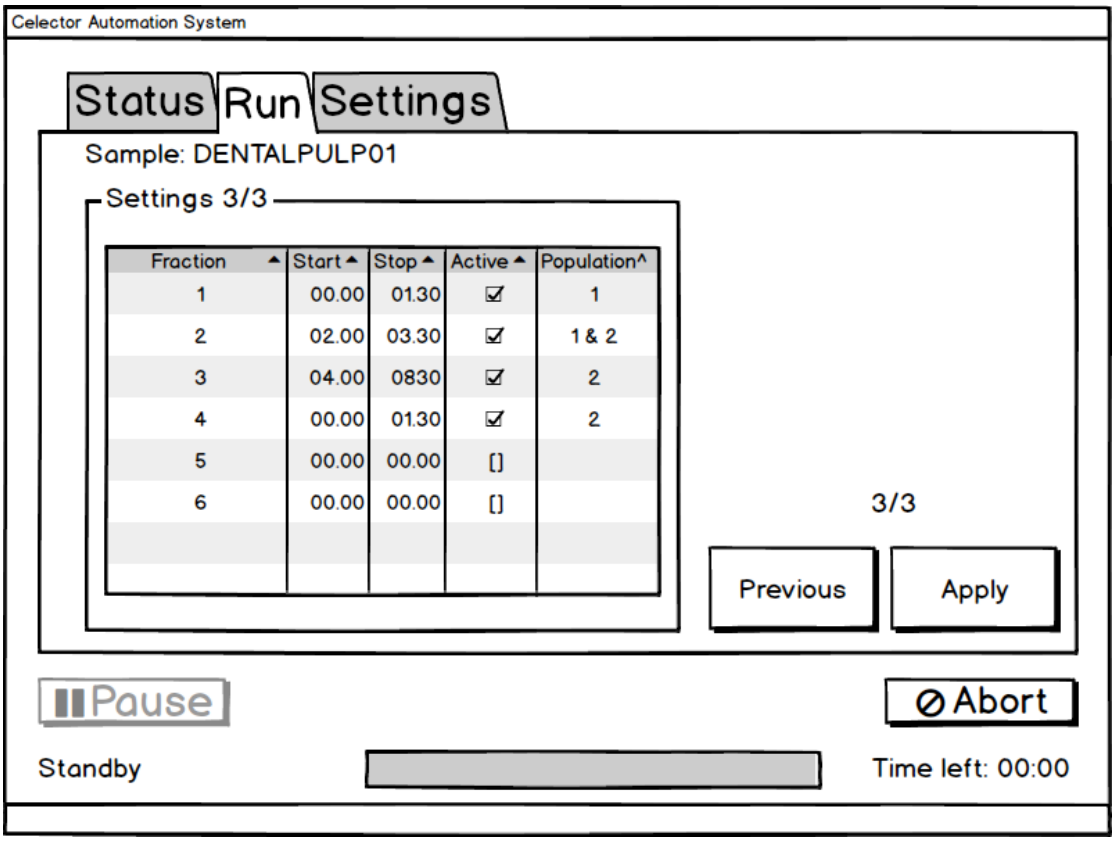
- The number of these cell populations.
- The size of the cells.
- If the cells are adherent or not.
- The volume to be injected.
- The central flow.
- The number of fractions and their populations.
- The type of test tubes used for the collection (15 or 50 ml).
- Possible stop flow and its duration.



Figures 2.20: Run Settings 1/3



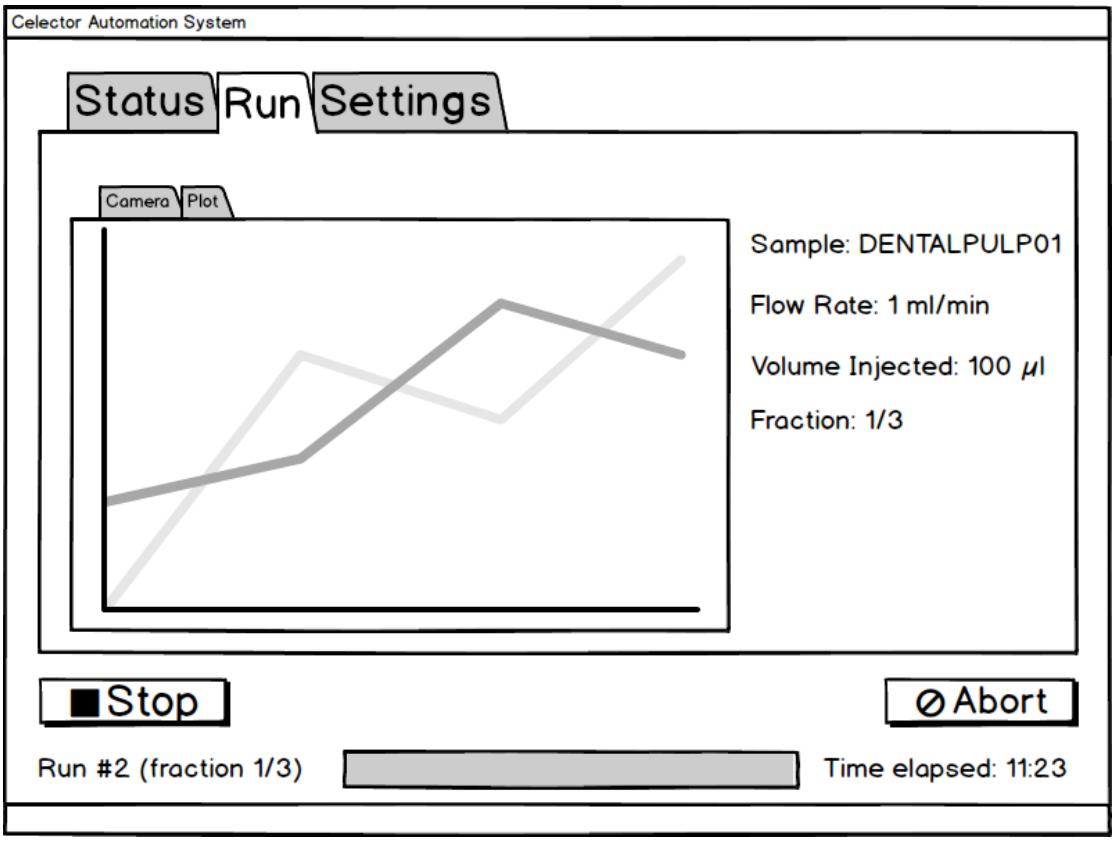
Figures 2.21: Run Settings 2/3



Figures 2.22: Run Settings 3/3

During the separation the camera is connected and the user can switch on the display from between real time visualization and fractogram like uotput.

The data relating to the separation and the separation curve are stored and available to the operator for later elaboration.



Figures 2.23: Running

5b) Fraction collection

Each fraction will be collected in a test tube, from 15 to 50 ml depending on the option selected by the user.

5) Washing

Two washing procedures are available, in reason of the proprieties of the processed samples: light and heavy cleaning.

Chapter 3

Development of Celector®

With the aim of bringing to the market a technology which marries the needs of the future users, and the industrialization requisites, I paid particular attention to the “heart” of the technology, which is the fractionation channel. The latter should let cells viable and not stressed by the separation process, and most of all, boast a high cell recovery in order, for different reasons:

- reduce the numbers of consecutive runs for fractions accumulation

- analysis and further characterization of all populations in the sample

- let the sample untouched, allowing their use in cell therapies

- process the higher amount of cell both in reason of high cell number required for therapies and of the separation of representative parts of the whole sample.

The new channel configuration implements a sort of fluidic guide in order to obtain a high cell recovery and avoid the contact of cells with the lateral walls of the fractionation capillary channel. Celector differences from the public-knowledge device used for NEEGA-DF analysis (Gr-FFF) both in the fractionation channel for the layers assembly and in the fluidic system. The Gr-FFF separation channel is a thin layer trapezoidal shaped, where

the mobile phase is introduced into one of the two triangular points, flows through the whole length of the channel and exits from the opposite triangular point. Inside the separative channel, the mobile phase presents a parabolic flow profile, not only in the channel thickness where it provides the separation process, but also in channel width meaning that the flow velocity at lateral walls is naught. When samples are introduced into this separative channel they are subjected to the parabolic flow and they raise the lateral walls where they aren't subjected to any velocities which should carry it to the outlet of separative channel, thus a part of injected cells are "entrapped" at the lateral walls, with a consequent low cell recovery (no higher than 50-60%).

The new separative channel design implements two lateral fluidic surfaces with a lower flow rate, acting as an external fluidic guide from the separation surface, that allows sample not to arrive at lateral walls and remaining always in movements (Figure 2.1). External flows are parallel to the separation surface so they are not considered factors acting for focus cells (like a flow cytometer) because there is no single-cell focalization. Moreover the cell separation profile is better defined and closer to a Gaussian peak profile, indicating a more efficient separation process.

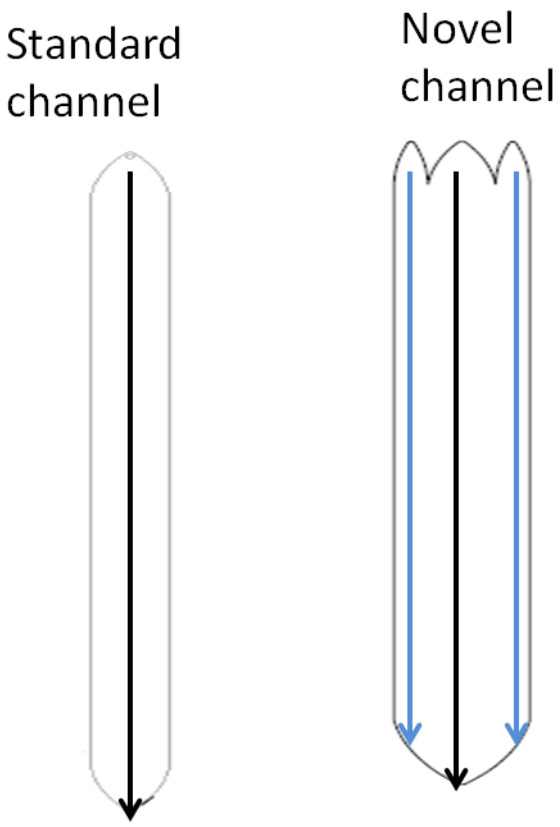
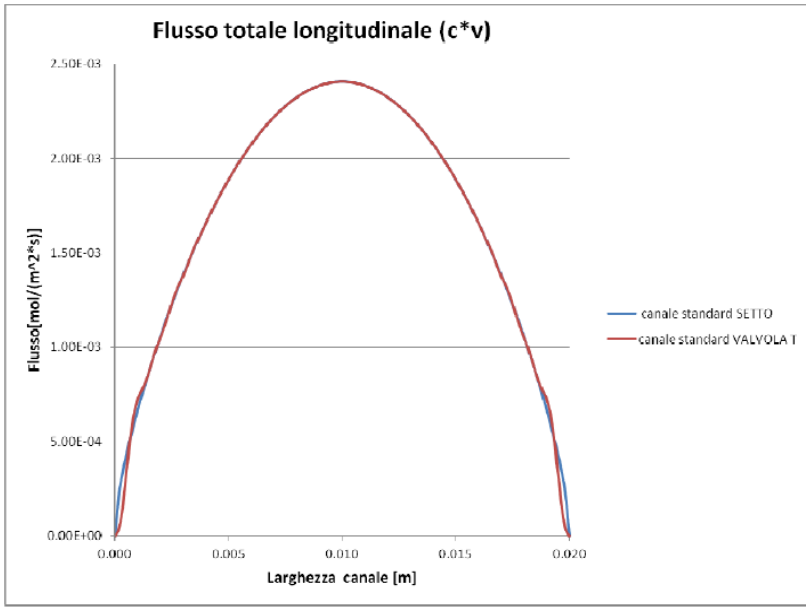


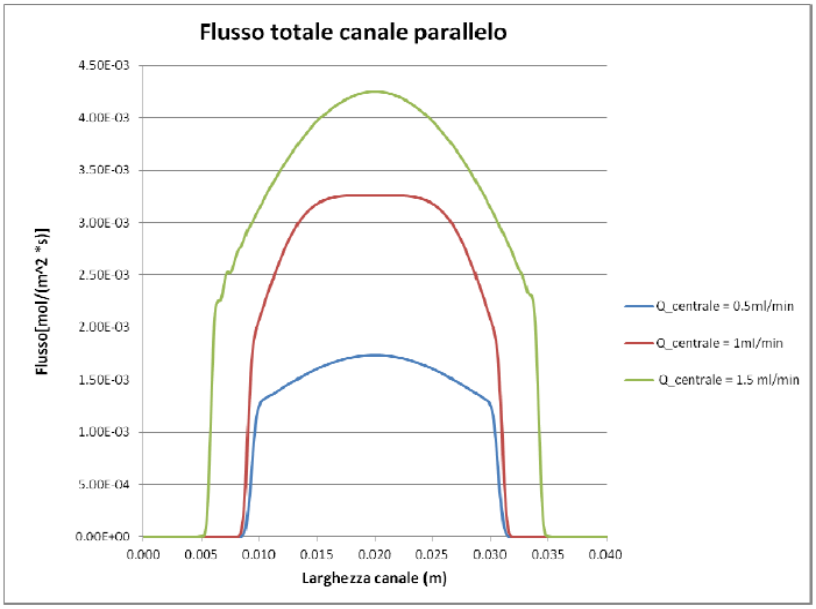
Figure 3.1 : Previous fractionation channel (left), current and patented fractionation device (right)

The Finite Element Method (FEM) analysis of the flow profile inside the separation channel, performed with different geometries and simulated with COMSOL Multiphysics 3.5, allowed to match the optimal geometry with few tests.

The result of the FEM analysis (Figure 3.2) shows the conservation of the lateral fluidic guides at different flow rates of the central separation flow for the new geometry.



(a)



(b)

Figure 3.2: FEM output: (a)Total flow profile of the standard channel (b) (a)Total flow profile of the new “parallel” channel at different flow rates of the central main flow.

I also optimized the injection system and position, implementing an in-flow injection that increases the recovery (adherent cells are not strongly injected against the accumulation wall of the channel) and decreases shear forces resulting in a lower stress of cell samples. The injection system was crucial in defining the fluidic asset. Traditional Gr-FFF injection system is composed by a T-valve and a syringe: the injection of the sample is completely performed by hand.

Biologists believe that the standardization of the separation protocol, in particular during the first steps of sample preparation or treatment, is always more requested by cell manipulation institutions because it's decisive for successive phases both for research and medical issues. Due to the poor amount of stem cells in a biopsy, the standardization of the collection and recovery procedures became essential, in particular during the sample characterization phase.

Citing the Journal of Cell Biology "…the integration of the various processes will be required in order to achieve a clinically relevant product through a regulated and controlled bioprocess that is reproducible, standardized, automatable (when needed), integrated and certified. …"[66]

In Figure 3.3 is shown the Gr-FFF instrumental setup with the T valve injection system.

[66] Vol. 4(4), pp. 40-52, December 2014, DOI: 10.5897/JCBG2014.003

Figure 3.3: NEEGA-DF instrumental setup with two fractionation channels working under laminar whood (left), injection system by T valve (right)

This injection system is not reproducible, “lands” the sample against the accumulation wall and scatters the suspension in the whole width of the separation volume causing loss of cells, presents high dead volumes and introduces air inside the channel compromising the separation. Moreover the method operated by biologists for the cell counting Burker chamber) introduces further uncertainty about the number of cells injected, or better both injected and recovered cells. By the way the error is higher on the injected cells because the same volume of the whole solution prepared is analyzed through the Burker chamber (usually 0.01 ml), but the volume of the whole solution is different: the volume of the cell suspension to be injected and separated is grater (usually 1 ml) than the one to be counted for the recovery evaluation (usually 0.1/0.2 ml)

$$\text{Concentration} = \frac{\text{Number of Cells x 10.000}}{\text{Number of square x dilution}}$$

The major Burker chamber Producer (Celeromics) asserts: "*Errors in the range of 20%-30% are common in this method due to pipetting errors, statistical errors, chamber volume errors, and errors from volume of simple introduced into the chamber. Even though, the Burker chamber remains the most widely used cell counting method in the world*".[67] [68]

To overcome these critical problems, both biotech and engineering aspects were considered: the first revising the biotech method in reason to reduce the steps "out of control" for the determination of cell concentration in the solution during its preparation; the second in reason to think the layout definition starting from the fractionation and the injection systems. In particular, part of the injection was included inside the layers of the channel, and then controlled by a stepper motor peristaltic pump, reducing systematic errors. This strategy dramatically decreases the formation of air bubbles during the separation, minimized the dead volumes removing the run-to-run volumes accumulations, standardized the injected volumes thanks to the accurate steps of the motor and the particular capillaries format and configuration.

The result of the optimization of the channel shape, the lateral flow rate setting, and the injection asset and method are shown in Figures 3.4, 3.5, 3.6, 3.7, 3.8, demonstrating the

[67] Strober W (2001). "Monitoring cell growth". In Coligan JE, Bierer BE, Margulies DH, Sherach EM, Strober W. Current Protocols in Immunology

[68] USA: John Wiley & Sons. p. A.2A.1. doi:10.1002/0471142735

absence of contact of cells (both adherent and in suspension cells) with the lateral walls of the new fractionation device. In the Figure 3.4 the positions of observation relative to following pictures are laid out.

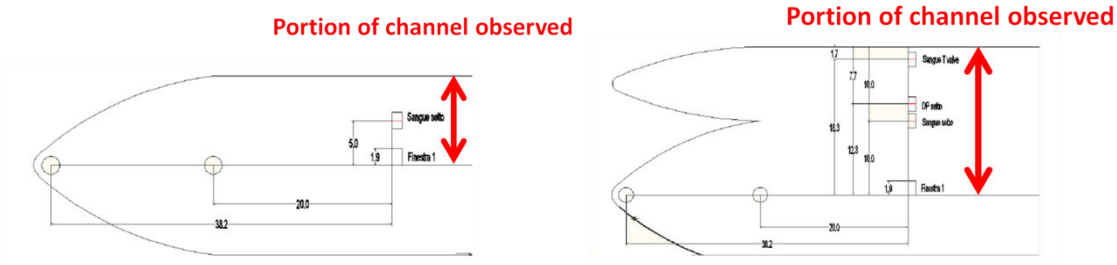


Figure 3.4: Observation positions on the standard channel (left) and the new channel (right) for the evaluation of the best fluidic regime of later guides and the injection protocol.

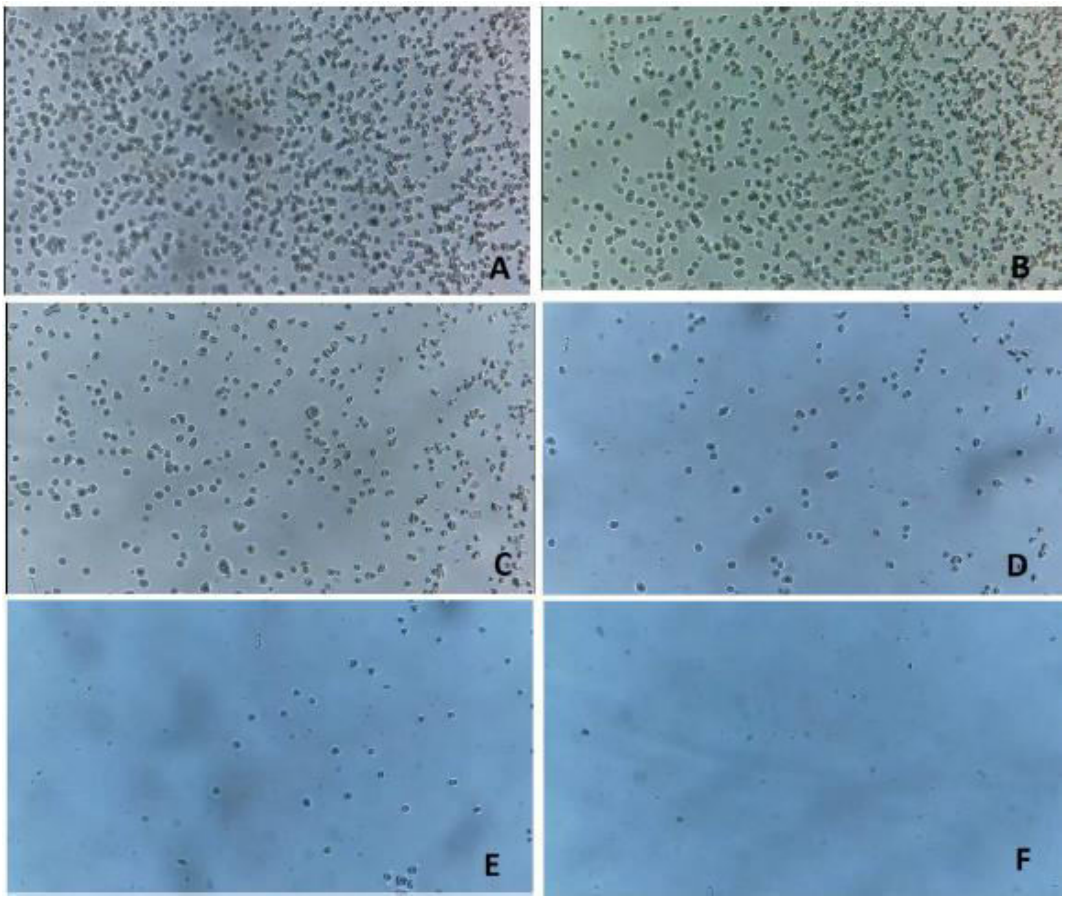


Figure 3.5: Sequence of pictures from the middle(A) to the edge (F) of the new separation channel. Dental pulp MSCs (adherent) injection.

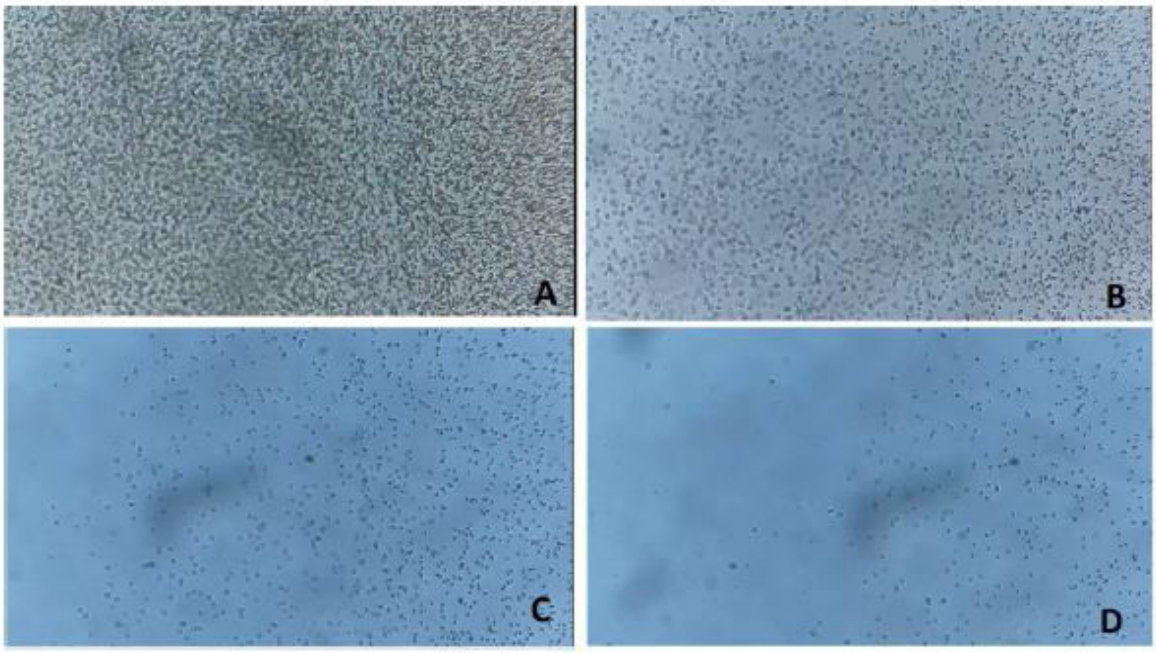


Figure3.6: Sequence of pictures from the middle (A) to the edge (D) of the new separation channel. Blood (in suspension) injection.

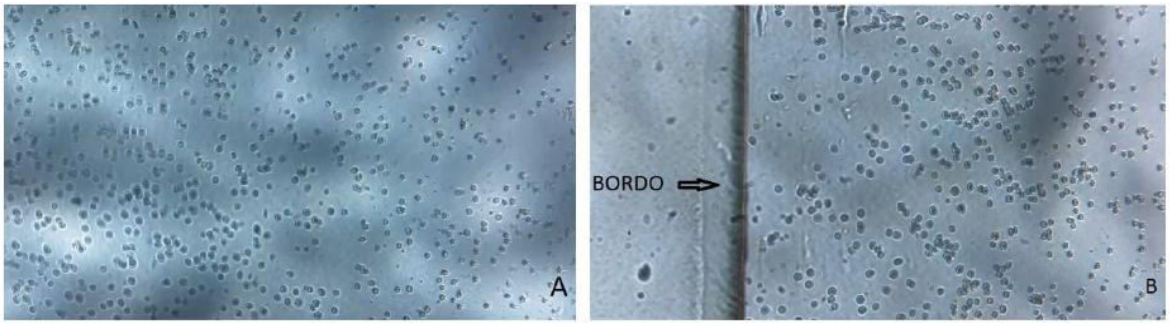


Figure3.7: Sequence of pictures from the middle (A) to the edge (B) of the standard separation channel. Dental pulp MSCs (adherent) injection.

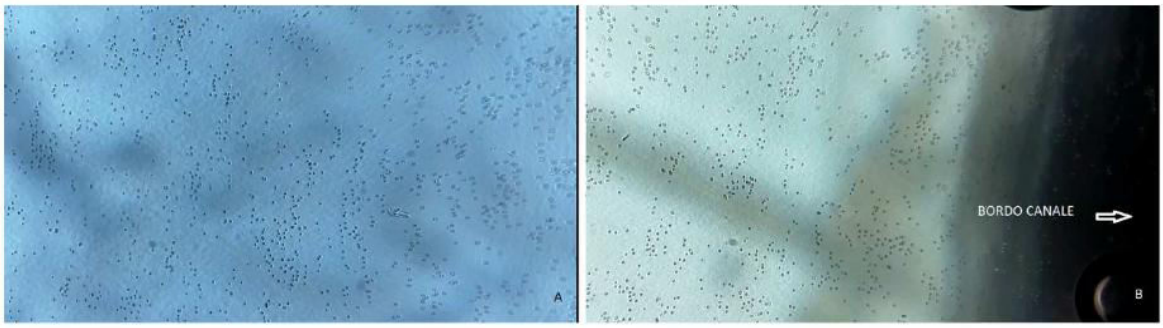


Figure 3.8: Sequence of pictures from the middle (A) to the edge (B) of the standard separation channel. Blood (in suspension) injection.

Combining the considerations on the channel geometry and the injection system, in order to get to an automated product, the composition of the multilayer separation device was adjusted including part of the injection between the layers.

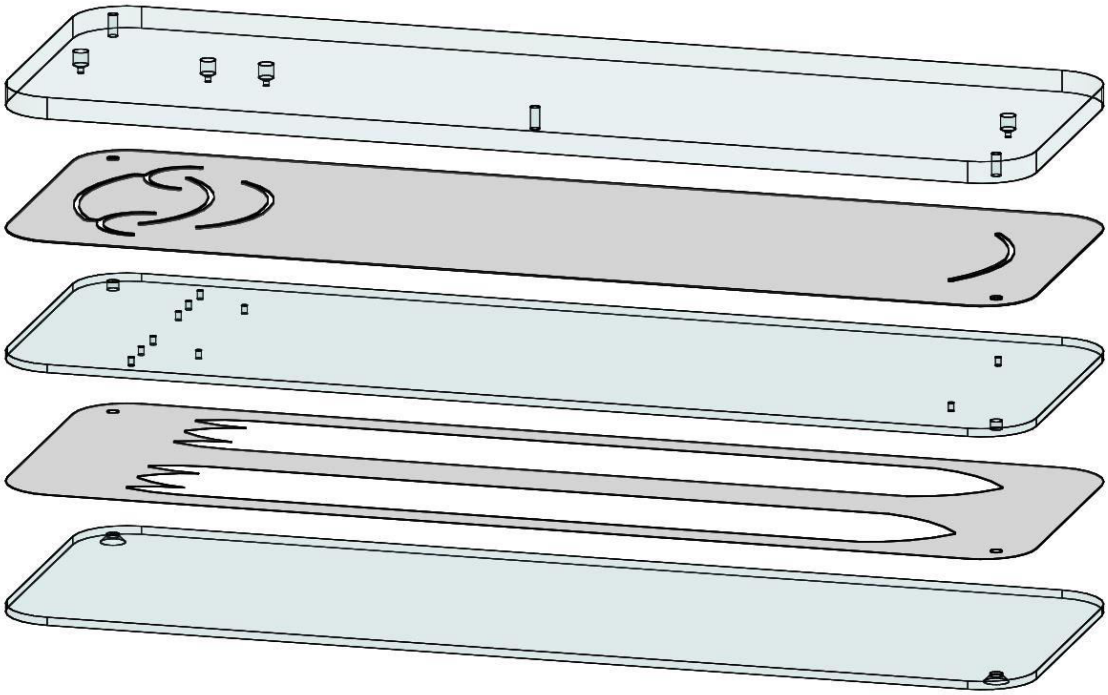
The new device presents five layers stacked together to give the hydraulic circuit.

Proprieties of layers are described in the Table2.1 and figure out in Figure 2.9

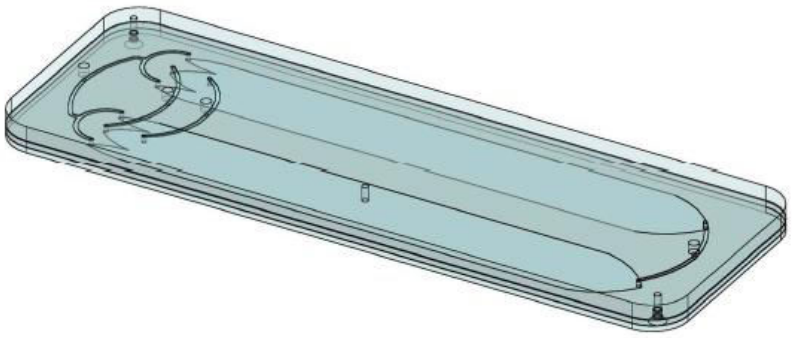
Layer	Thickness	Material	Function
1	8 mm	PMMA	*Inlet and outlet of liquids and sample*
2	0.25 mm	PMMA	*Controlled volume of injected sample (0.1ml)*
3	3 mm	PMMA	*Depletion wall*
4	0.25 mm	PMMA	*Lateral walls*
5	3 mm	PMMA	*Accumulation wall*

Table3.1 : Layers composing the fractionation device.

Layer 3,4,5 are responsible of the cell separation, Layer 1 and 2 are responsible of the injected cell amount standardization and in-flow injection not directly on the accumulation wall.



(a)



(b)

Figure 3.9: Exploded (a) and assembled (b) multilayer channel

The separation methods were defined in a few months, first to merge the results with the technological development and second to compare the performances respect to the standard technology NEEGA-DF.

The brilliant ideas and the considerations in depth of the results produced by the research team, blossomed to an accurate experimental design that allows the simultaneous development of the different parts of the instrumentation with an “overviewed” approach, adjusting step by step tools and methods.

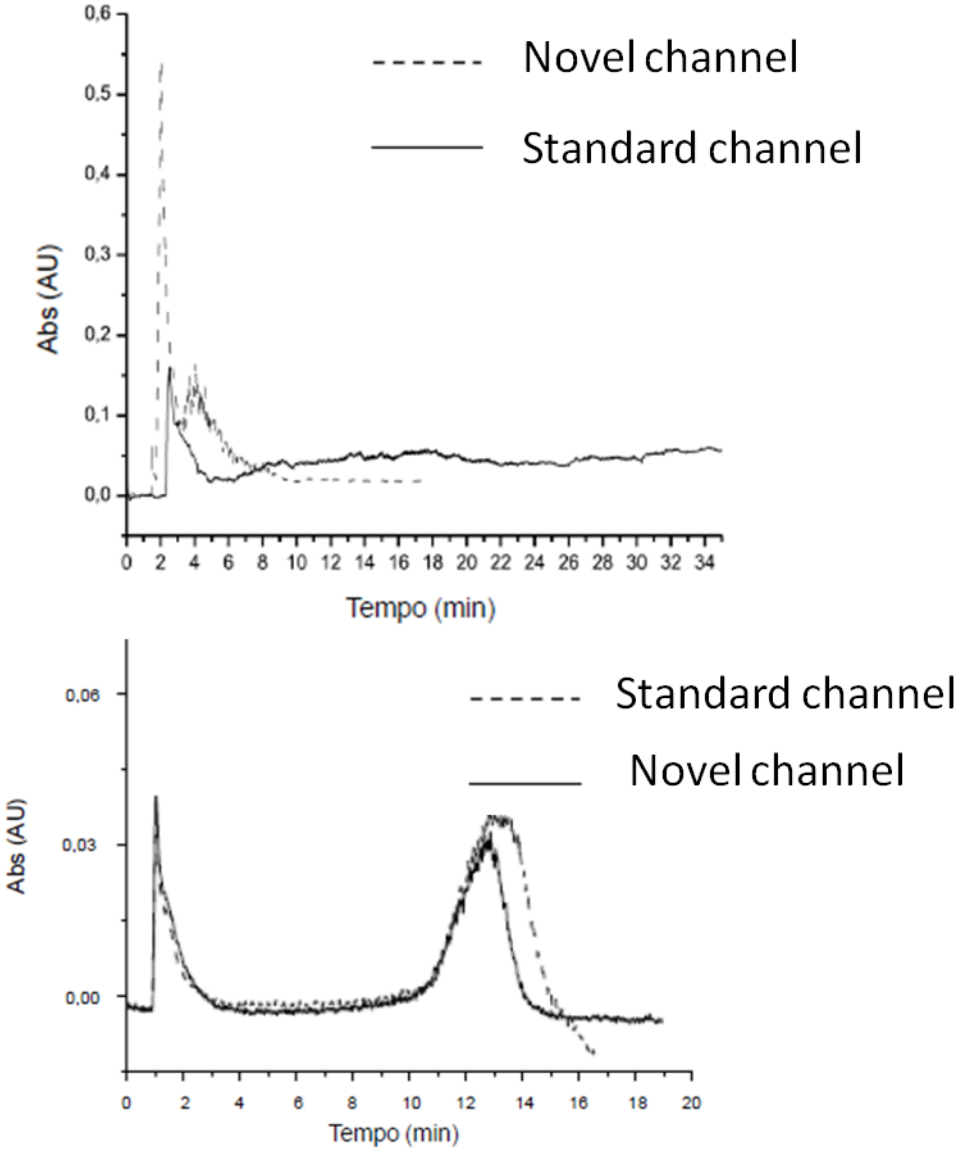


Figure 3.10: Overlap of separation profiles of mesenchymal stem cells performed with standard (___) and new (---) device using the NEEGA-DF method (above), overlap of separation profiles of red blood cells performed with standard (----) and new (___) device using the Gr-FFF method (below)

One of the first results was the filing of a patent application for the new device and separation methods. The patent application in Italy (PA94250IT, “Dispositivo per il frazionamento di oggetti e metodi di frazionamento”) was performed in the year 2014, while the search report, its response with claims revisions and the filing of the patent PCT application (PCT94250) belong to the year 2015.

At the same time was carried on the definition/revision of materials composing the fluidic system. The materials were selected throwing in together the medical device IIb compliance, the cell manipulation requirements for best separation results, and the industrialization requirements as material availability, cost, quantity for lot, order fulfillment, manufacturing process feasibility/repeatability, suppliers reliability. From the beginning the hydraulic circuit as thought to be used as disposable (replaced also at every run if necessary), avoiding any risk of contamination. Accordingly its running is entrusted by external tools along the whole hydraulic system, able to manage the flow and the cell suspension without any contact with them.

The best compromise in materials and configuration developed by the research team is illustrated in Figure 3.11 and some particulars are shown in Figure 3.13.

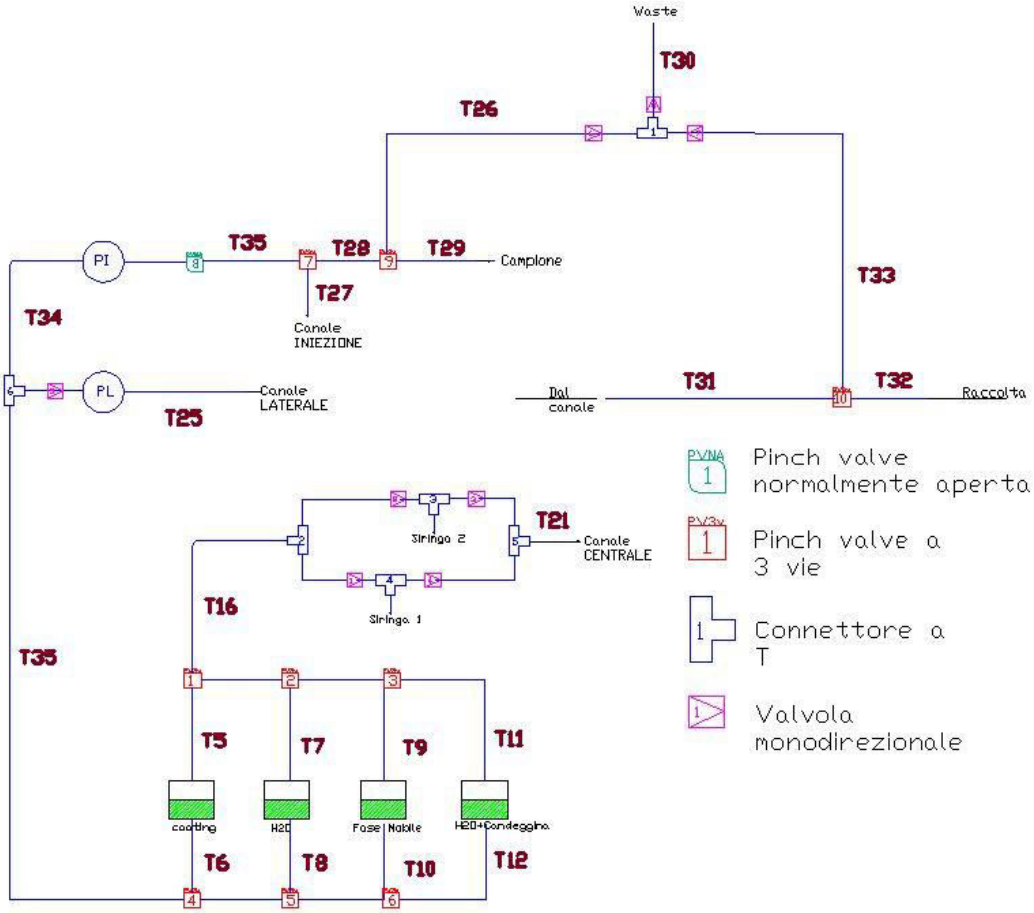


Figure 3.11: Hydraulic circuit scheme (channel not included)

The hydraulic circuit is composed by the components listed below:

- Silicone tubes
- Fittings and adaptors for tubing connections

- Syringes for pumps
- Falcon for collection of fractions
- Check valves

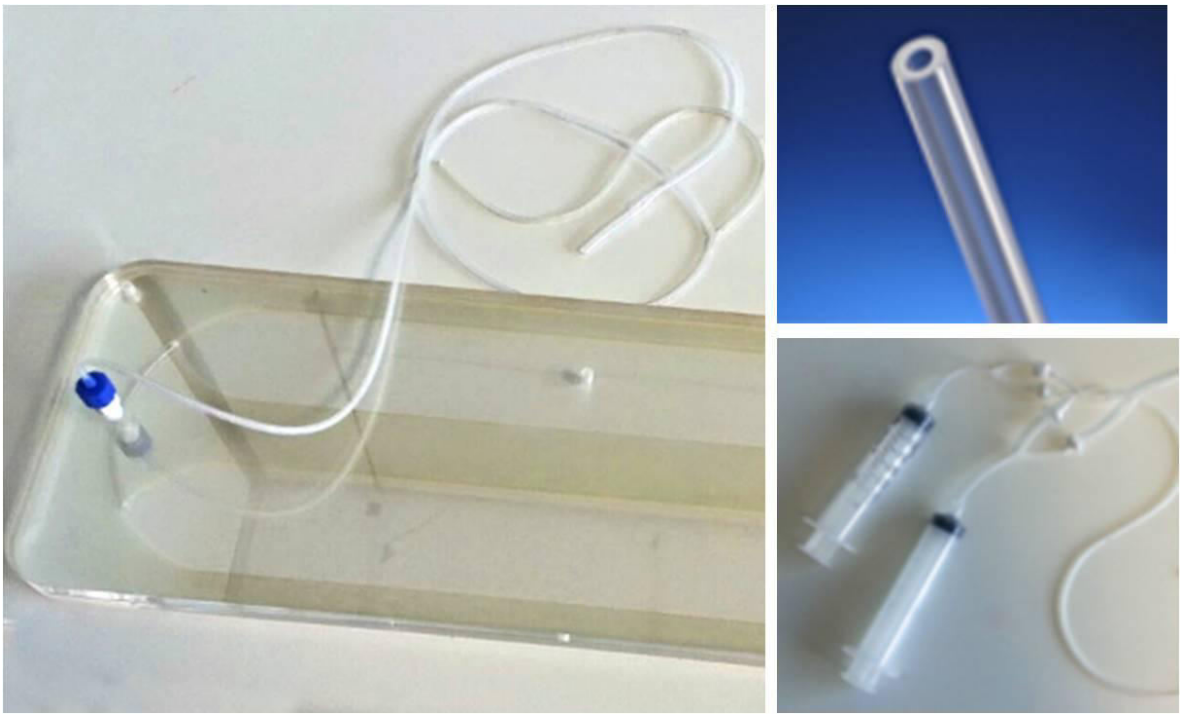


Figure3.12: Particulars of the hydraulic circuit

To complete the hydraulic section, it had to be defined how the seal of the stacked layers constituting the channel would be guaranteed. This topic represented the “technological challenge” of this project, the unsolved trouble which is gripping FFF researchers and experts for decades. Our results untie the belief of “unfeasibility”, but have is useful only for the start up production with small batches, and needs to be industrialized before the

large scale commercialization that will make available Celector® for R&D/medical/clinical purposes.

Conventionally the hydraulic seal of channels for FFF separation is acted by a series of bolts distributed along the thin layer perimeter and tightened at a specific force by a torque wrench, which depends from a series of parameters (layer material, thickness, width, geometry,…).

The new design of the channel doesn't allow the use of bolts because the force wouldn't be distributed homogeneously on the surfaces and the deformations originated from the tightening force would compromise the parallelism of the layers originating leakages.

I explored many solutions; some of them were tested with the intention of replace bolds passing to a "light" device that replace the "heavy" mechanical arrangements, the latter inappropriate for cell manipulation and for disposable usage.

The technological challenge was very hard.

Some examples of bolding and press-like sealing systems are reported in Figure

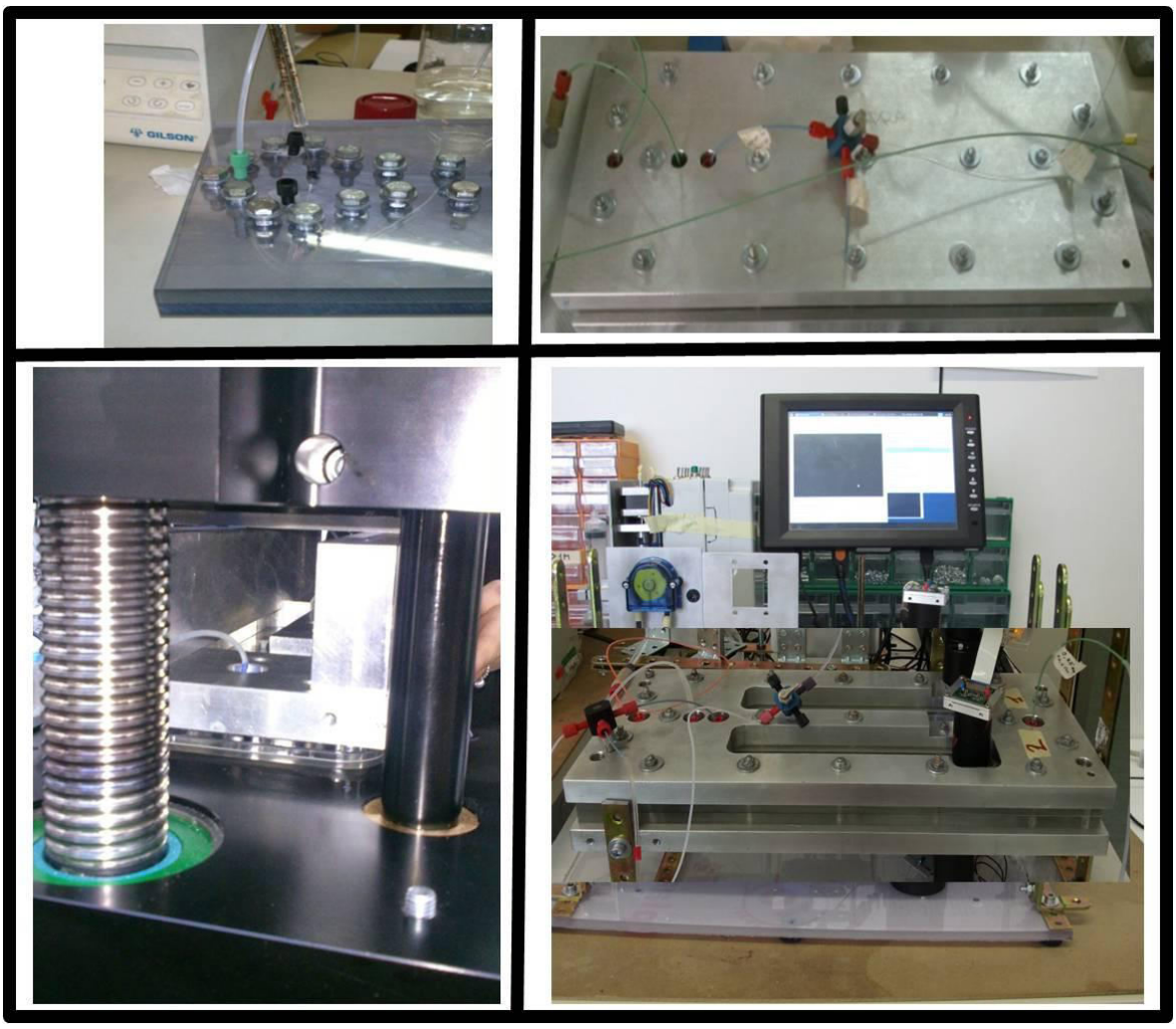


Figure3.13: from the left: bolds, Aluminum plates to uniform the force on the whole channel surface, engineered press-like solution, Aluminum plates which allow the detection.

Others manufacturing solutions failed due to the numbers of the layers: too many or too few.

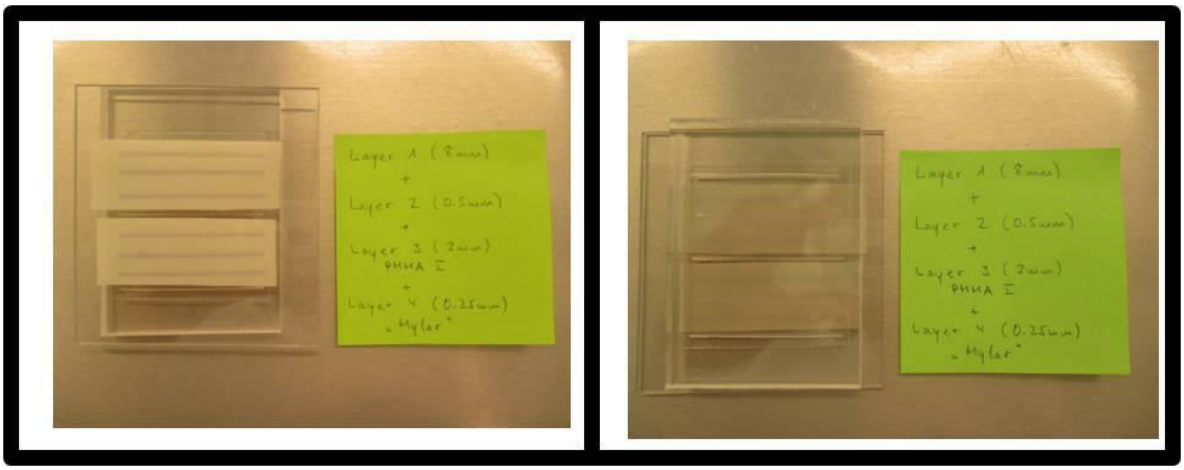


Figure 3.14 : Laser welding tests with different materials. 4 of 5 welded layers

Figure 3.15: Milling machining tests. 1 of 2 layers treated: on the left the assembled 2 layer channel, on the right the picture under microscope of the treated layer.

In the Figures above are exampled two representative cases of welding technologies and processes tested to replace the external mechanical tightening system in reason to obtain a self sustained channel easy-to-use for future costumers.

At now there's an only procedure that guarantee the hydraulic seal of the channel in working conditions, even if the solution isn't ndustriaslizable on large scale. Preliminary tests have been performed to optimize the production process for the first lots intended for beta testing phase. Meanwhile new tests on different techniques more appropriate to large scale production, are scheduled and will be worked out during the beta testing, receiving advantageously the first feedback of testers.

The working solution implements a Double-sided adhesive tape, which replace the thin layers of the separative channel.

Layers 2 and 4 are so replaced by a tape meting together upper and lower layers without significant loss of the parallelism also under stressed conditions. Two materials replacing the thin layers were tested, with good results in the polyester tape assembled device.

Figure 3.16: Prototypes of self-sustained channel. The thin PMMA layers are replaced by (1) Acrylic foam and (2) polyester biadhesive tape layers of the same thickness respect to PMMA layers.

The device assembled with polyester tape overcomes to all working phases of Celector® without any support, The separation proprieties were studied both with standard particles with a density similar to the cell one, and with MSC cells in order to adjust the manufacturing and processes of production phases, achieving the firsts lots of self-sustained channels to place on the market.

Two revisions were tested improving both the separation features and the behavior in the different phases of working. In particular, began the study about the interaction with the cleaning solution and the toxicity tests in order to validate the biocompatibility.

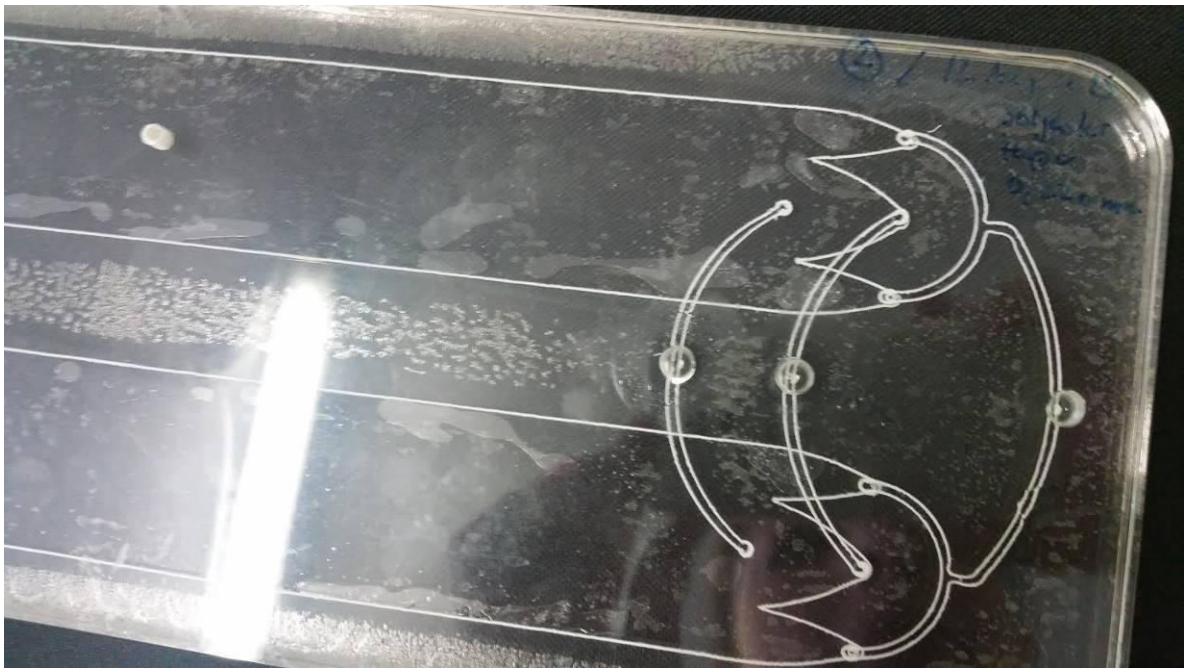


Figure 3.17: Sedimentation of salts on the edges of biadhesive layers

We noticed that the adhesive tape is different after some separation runs. The edge of the tape is altered and it seems that salts into the separation buffer and cleaning buffer sediments on the edge of the tape. This leads to holes obstructing and prevention of the liquids flow.

A first series of improvements about the manufacturing process was performed in reason of these considerations highlighted by the supplier and discussed fort better results (Figure 3.18):

- Plastic layers get electrostatically charged by removing the protective cover film and dust particles or plastic residues of laser cutting accumulated on the surface.

- The screw connections already contain a lot of residues produced during the previous threading process
- The PMMA plates are deformed during the laser cutting procedure that melts the edges through the heat released

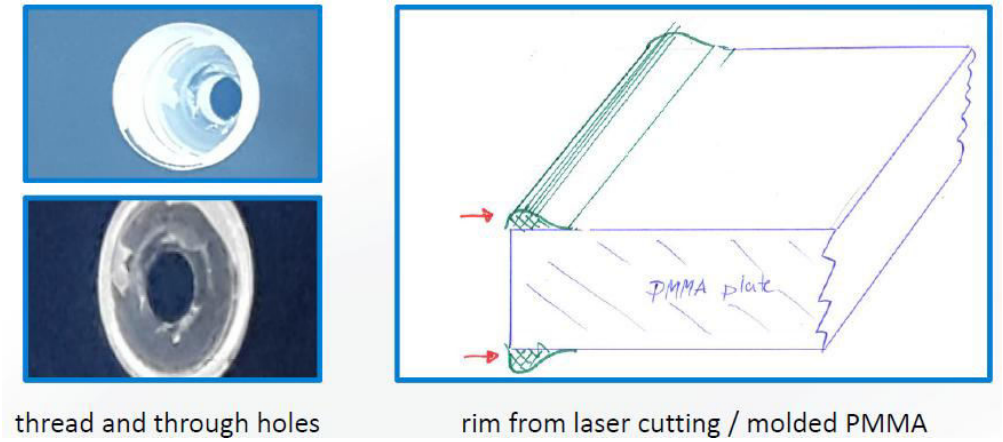


Figure: 3.18 Problems observed during the assembling, caused by previous manufacturing.

So we considered to add two steps before the assembling:

1. Edge smoothening
2. Ultrasonic cleaning in demineralized water

Four channels were assembled implementing the discussed procedures.

Two of them were designated to the separation method validation; one each was given over to engineers and to biologists. Main goals for each concern:

1. Stability of the fluidic system, material stress resistance, software adjustments, hardware components evaluation/replacement/placement, repeatability of the method, shelf life estimation, dead volumes tests, recovery and counting software tests, draft of the certification agreements and risk analysis annexes.
2. Sterility subsistence, easy-to-use evaluation, interaction with cells both “in flow”/“in flask” conditions, interaction with biotech instrumentation equipment and ease of access to labs (e.g. no ground in sockets of laminar hoods and of the most of labs meant get shock every time a conductive material was touched, obviously this hampered the use of Celector till the problem was identified and solved), optimizing the timing of every phase, cell recovery,

The fluidic characterization of Celector® was performed studying different aspects with different separation protocols. For example, we set a sequence of experiments in order to determine the repeatability of separation with the new fluidic circuit using PMMA standards. PMMA microspheres are an optimal alternative to cells, because of their similar density and dimensions (0.015 mm) respect to Mesenchymal stem cells.

This characterization is performed in order to confirm that the separation proprieties are suitable for the separation of cells and the separation is repeatable.

In Figure 3.19 are shown the overlap of separations performed in different days. The retention times of aggregated and single particles are not different in every run, demonstrating the stability of the fluidic system.

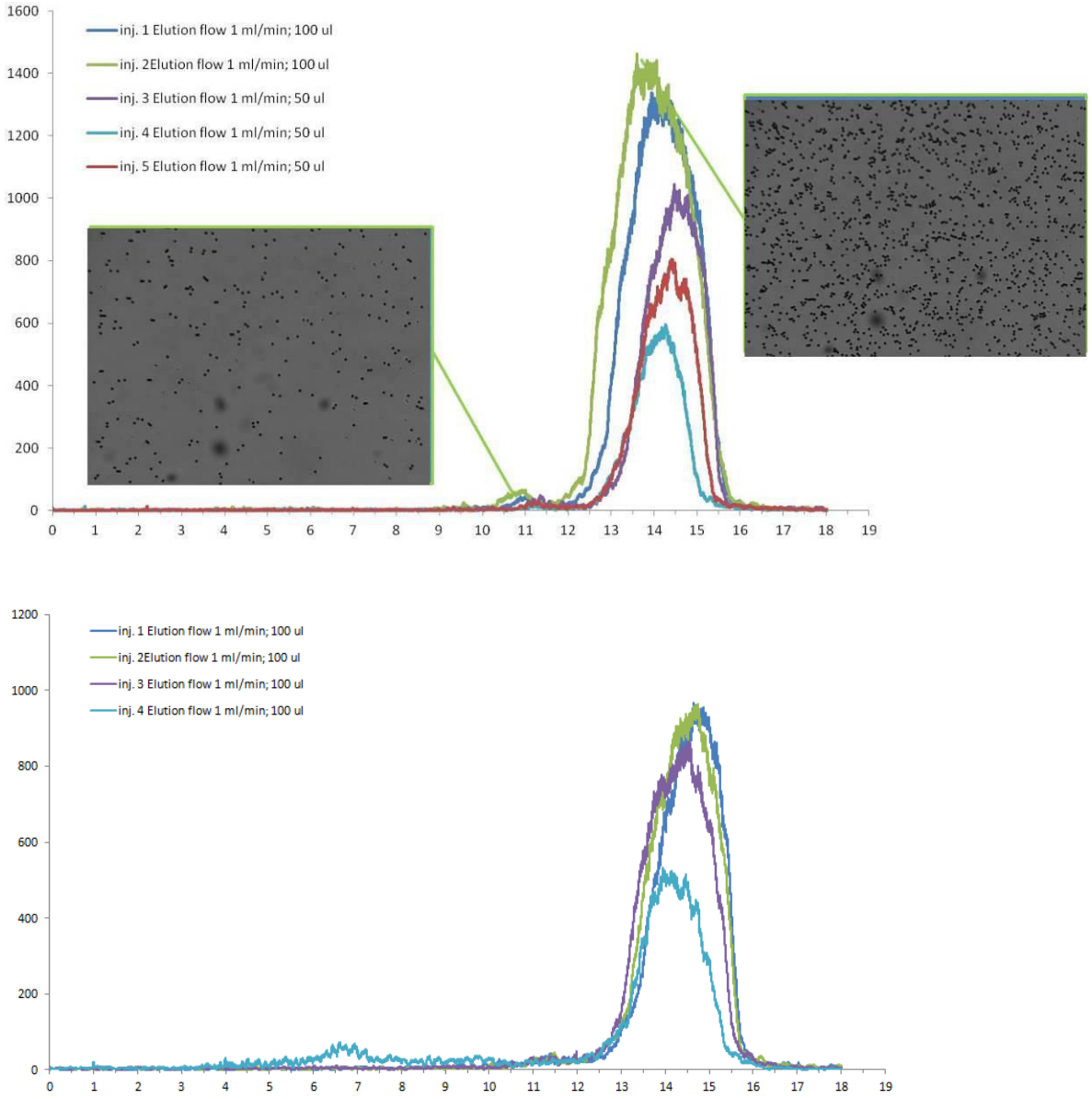


Figure 3.19: Overlap of separation profiles of PMMA particles in different days

Biological validation of the sealing solution was performed in parallel.

Toxicity tests can be chosen on different basis. We need to do specific and punctual tests, with a good reliability and short timing. We chose the **Direct Contact Method**:

1. A near confluent layer of fibroblasts are prepared in a culture plate (24 wells with HOS cells plated at 1.500 cells/well)
2. Old cell culture media is removed
3. Fresh media is added (IMDM + PS 1% + FBS 10%)
4. Material being tested is placed onto the cultures, which are incubated for 24 hours at 37 degrees Celsius (biadhesive 2mmx1cm)

The plates, prepared in duplicate to avoid contamination or confluence problems, are made by these specifications:

- Positive Control (C^+ cells in culture media)
- Negative Control (C^- cells + 30% bleach in H_20) → we assume that was the contamination with bleach the cause of cell death in the previous experiment
- Sample + biadhesive washed with sterile H_2O
- Sample + biadhesive washed with 30% bleach and sterile H_2O

After 24 hours at 37 degree Celsius, cells are as shown in fig. 3.20: the biadhesive shows no toxicity for cells, both biadhesive washed with only sterile water and washed with bleach. The material is floating and not adherent on the wells. Positive control (cells in

culture media) is suffering more than the sample with the material. Negative control is made by cells in culture media with 30% bleach and they are dead (as we want).

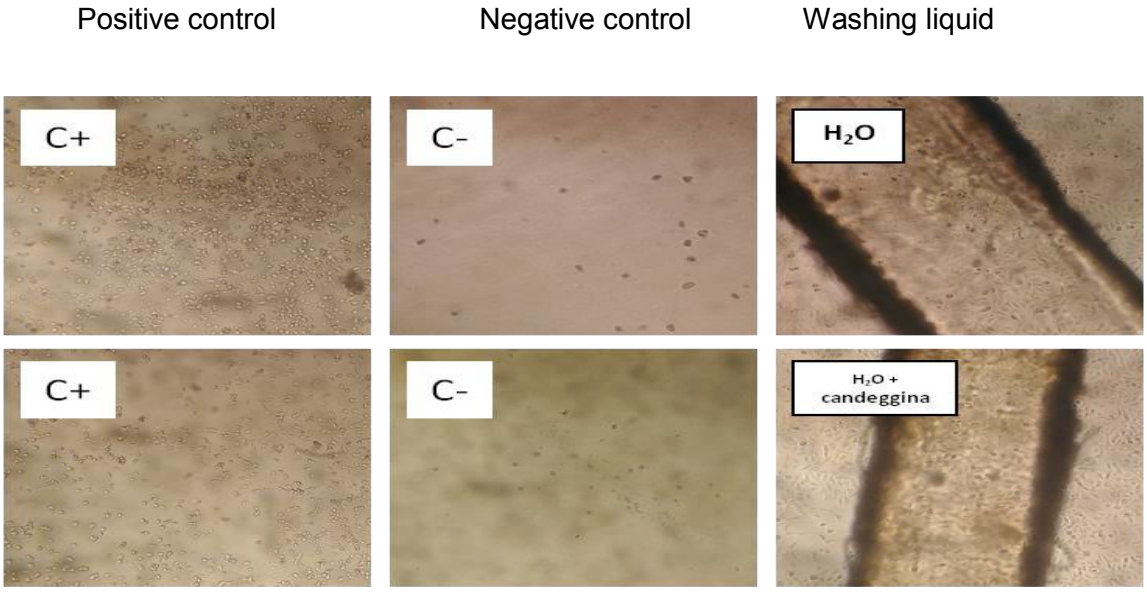


Figure 3.20: Toxicity test results

Results demonstrate:

- The biadhesive is biocompatible.
- The biadhesive releases some molecules/substances promote cellular adhesion on plates

The detection system replacing the usual absorbance signal consists in the counting of cells during the separation.

The approach was to identify the concentration distribution function of the injected cells, along the width of the channel, so as to obtain the multiplicative factor that allows to extrapolate the total number of cells fractionated for each fractionation.

The procedure to derive the distribution function was:

- Consider the width of the channel consisting of two symmetrical halves A and B (Figure 3.21) in which it is assumed that the analytes are arranged in the same way
- Divide one half (A) in several regions of observation called windows. (Figure 3.21). The windows are near to the collection point f to ensure that the separation process is terminated, and then the number of cells counted corresponds to the number of collected cells or present in the collection tube

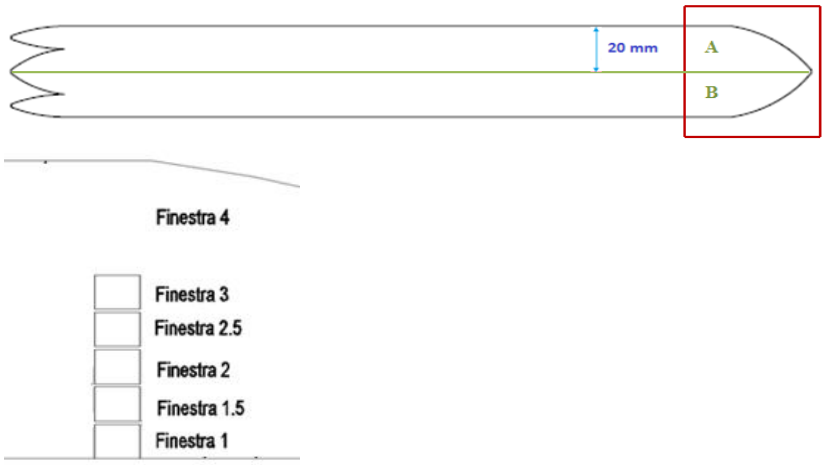


Figure 3.21: Partition of the channel in observation windows

- Take a sequence of pictures during the cell separation positioning the optical system in correspondence of the window.
- Move to the next window till the edge of the channel
- Once determined the distribution of concentration along the width of the channel which considers the percentage of cells crossing every windows, the rough data

(number of cell counted) will be multiplied for the parameter find out from that function

The elaboration of the counting software is based on the image analysis normalized on the velocity of cells crossing the window in reason to count every cell only once.

The image analysis operates as described:

1. loading the image (previously scaled)

2. subtraction between the image and the background image (without cells)

3. erosion + dilation of the image obtained so to eliminate the edges

4. from color image to grayscale transformation

5. application of the threshold effect

6. Cell contours detection and perimeter calculation

7. Discard of inadequate particles having too high or too low perimeter (bubbles, impurities,…) thanks to a size operating filter

Figure 3.22: Borders detection during the separation

The software needs the diameter of injected cells for the calculation of the perimeter, so distinguish cells from the background, and to set the dimensional cut off.

Several versions of the counting algorithm were experimented but no one fitted the actual amount of processed cells, or better the parameter find out from the statistical analysis presented a too high error to be considered reliable.

The best result obtained, where the cell number was closer to the actual amount of processed cells gave the concentration profile as in Figure 3.23, and relative separation profiles (observed in the window 1) as in Figure 3.24.

This series of experiments was performed using Dental Pulp Mesenchymal Stem Cells

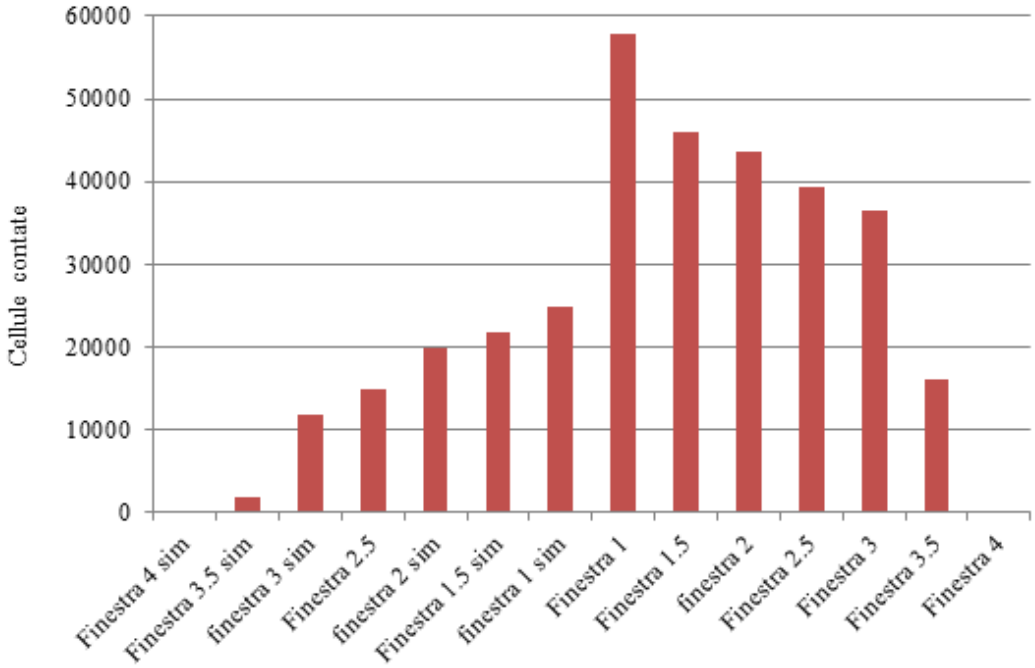


Figure 3.23: Distribution concentration profile obtained from the counted cells in every window

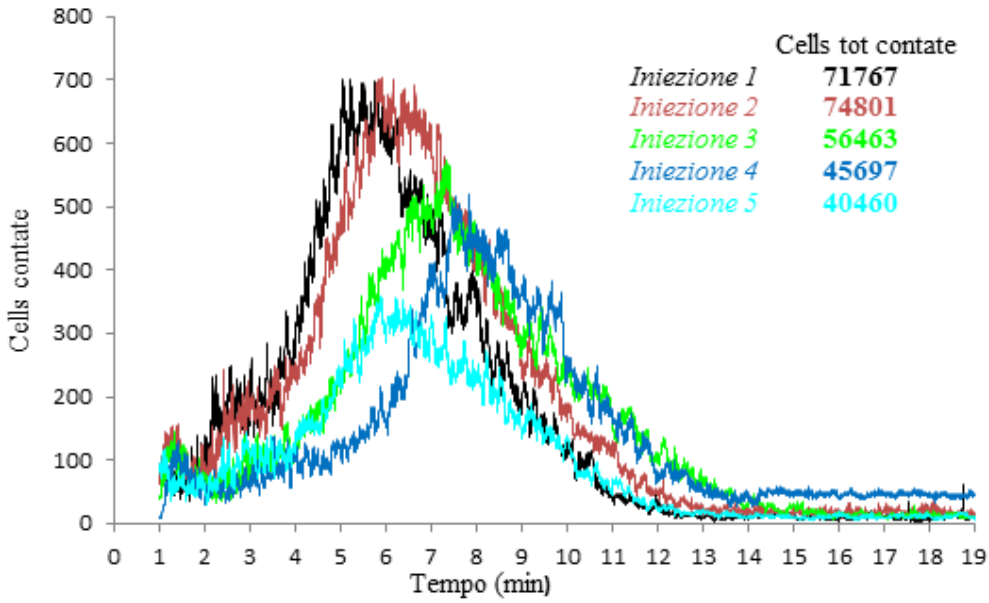


Figure 3.24: Separation profiles of processed cells registered in window 1

The average cell number counted by the software in every window is below reported in Table 3.2

Window	Avarage cell number
4 s	0
3,5 s	1900
3 s	11720
2,5 s	14785
2 s	19869
1,5 s	21826
1 s	24921
1	57838
1,5	46026
2	43498
2,5	39404
3	36584

3,5	**16098**
4	**0**
Total cells	***359129***

Table 3.2: rough data obtained from the counting software in every window exploring the whole width of the channel: windows from 1 to 4 are in the right half, windows from 1s to 4s are in the left half.

The trend of the distribution profile is not parabolic as predicted by FEM studies. The profile obtained is not symmetrical, probably due to the closing system (aluminum plates) which deforms the layers of the channel because of the punctual applied force creating an irregularity of distribution on the surface. This results in asymmetrical asset of the fractionation device.

Then considering as reference value the average number of cells counted in the window 1, the variation of cells counted with respect to the window 1 for the other windows was calculated normalizing the counted cells on the cell velocity and the number of frame registered by the optical system.

The sum of the variations for every window returns the value of the multiplier parameter. In this case he result of the total count is equal to 359.129, respect to the number of processed cells equal to 300.000. The counting software overestimates the count of about 30%.

In the end we decided to adjust the channel geometry in the first instance so to detect the totality of processed cells Figure 3.24.

Figure 3.24: Channel configuration for the detection of all the particles processed

This is a software simplification and, at the same time, a hardware complication, due to the grater linear velocity reached by cells entering the shrinkage. The hardware have to be replaced by with more performing tools, to allow the visualization of the observation windows through a brighter illuminator, the catching of defined pictures through shorter exposure time meaning a faster camera, which however needs to faster communicate with the microcontroller avoiding its overloading.

Actually this study has the only goal of settle the detection system, because the channel itself has a width range going from 40 mm to 2 mm, so the position of the detection system determines the percentage of the channel observes for the count.

In reason of these considerations will be planned experiments to define the right configuration.

The use of Celector® technology was made available for beta testing phase, during which will be used by cell processing labs, thanks to the development of the management software.

The software features:

- the interaction with the user via a graphical user interface (GUI) where the actions are performed through direct manipulation of the graphical elements.
- the full automation of the processes, controlled by personal computer that communicates with the Arduino implemented on board of the technology and with the PCB of the syringe pumps by a serial port RS232.

The automation software was developed outlining flowcharts describing every working phase: conditioning, running, washing and standby phases. With the possibility to choose “single” or “double” channel fractionator device.

An example of flowchart for the Running phase in Figure 3.25

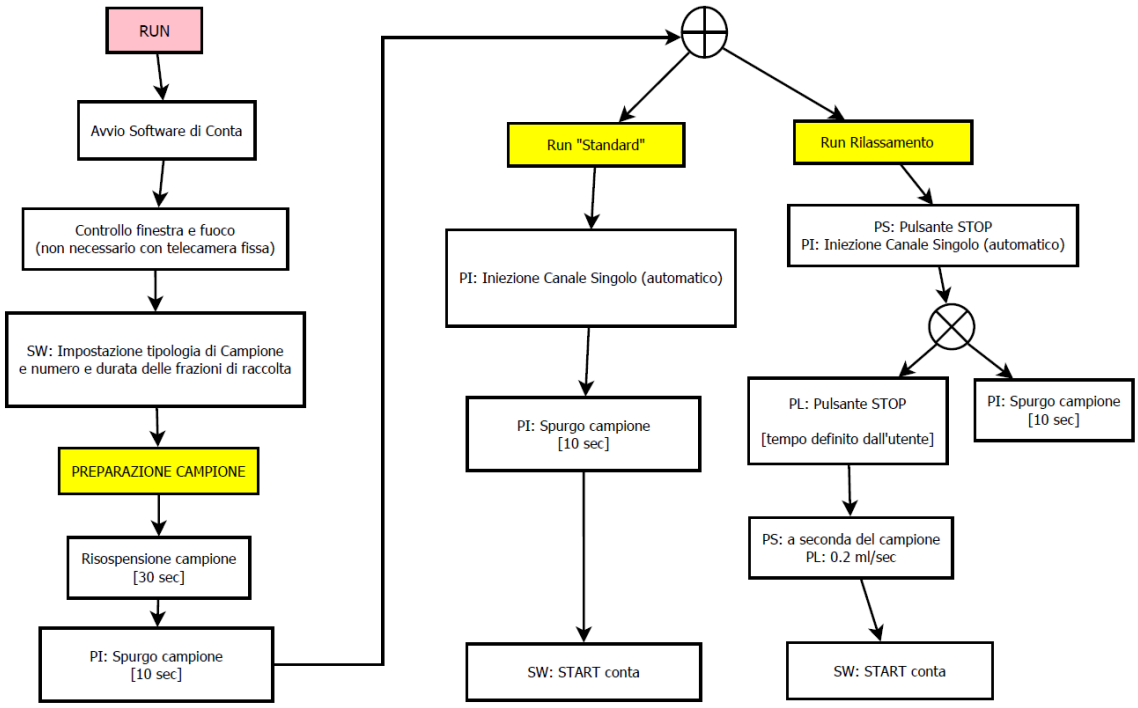


Figure 3.25: Flowchart of the Running phase, useful to drive the writing down of the Automation software and for the debugging.

The interface allows the not specialized personnel in using the Celector® through graphical icons and visual indicators. The outfit of the GUI realized is shown in Figure 3.26.

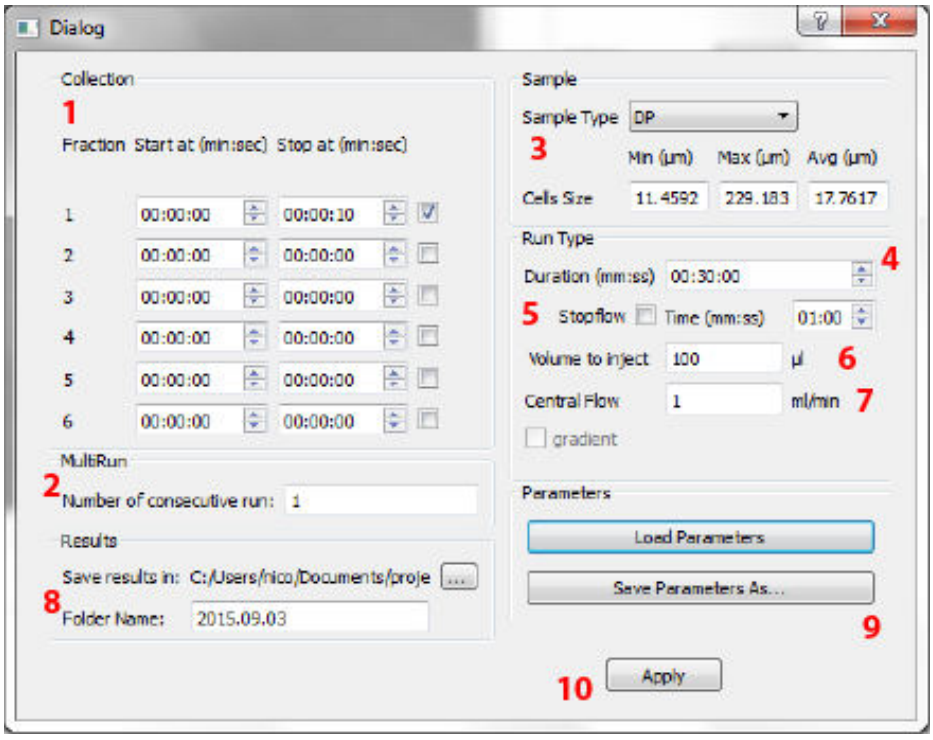


Figure 3.26: graphical user interface of Celetor®

Within this window it's possible to set:

1. the different fractions to collect (from a minimum of 1 to a maximum of 6). It's possible to set a no-collection time between two fractions.

2. the number of consecutive run

3. the type of cells and their parameters (default values are implemented and refer to tested samples)

4. the total time of the run so to return in the stand-by modes avoiding liquids wasting.

5. the "stop flow" analysis and its duration

6. the volume injected for every run

7. the separation flow rate actuated by syringe pumps

8. 9. saving favorites parameters and the desired folder

10. apply for the setting of the instrument

During the run it is possible to play both the movie of cells crossing the camera or the separation profile given by the counting software.

At the end of each run the data are saved to files in the folder and the graph is saved in two different formats (PNG and PDF) as well as the cellsdata file containing the individual count for each frame and the file out with the total count of cells.

Conclusions

I consider my PhD period as a real opportunity to bring my knowledge, my proficiency, my creativity, within reach to whoever might be useful. All the team played a central role reaching a fully function instrumentation to bring in manipulation labs. Many aspects are uder revision and development optimizing the whole system in the hope to reduce the testing phase, first implementi the right solutions paying attention to future customers needs.

The aim of the PhD is to act as a bridge between the academic world and the industrial one and the goal was reached.

The instrument developed during these years was tested also with biological samples, not reported in this thesis because not published yet. During the next years the beta tesing phase will produce final results to implement this work in a complete manual of a research device.

The excellence shaped by the University of Bologna stands out, once again, with an innovative product, thanks to the support of ALMACUBE which get by and coached the team, aiming to the formation and to the maximum efficiency oriented to business return and development.

The project reached very high levels and there is the need of an instrumentation so innovative

Compared to the market benchmark, you can then highlight the following innovative aspects:
1. EXCLUSIVE APPLICATION: the only instrument on the market able to select multipotent cells (e.g. Mesenchymal) without the use of markers.

2. NO HANDLING/MANIPULATION: cells not only are selected without immunolabeling, but also without any contact with the separating device: the cells are simply suspended / diluted in a physiological buffer solution (e.g. phosphate buffer, PBS).

3. EASY-TO-USE AND TIME OF USE: before being introduced in Celector® the desired number of cells, is only requested a centrifugation. It is not necessary the marking process, which usually takes at least 2 hours. The injection of the cells occurs through an automatic injection system easy-to-use.

The first prototy are the perfect way to explore the market and deep study the development strategy.