The immunophenotypical and functional characterization of ASC subpopulation in vitro

Peng, Qiuyue

DOI (link to publication from Publisher):
10.54337/aau473637566

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

THE IMMUNOPHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF ASC SUBPOPULATIONS IN VITRO

BY
QIUYUE PENG

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

THE IMMUNOPHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF ASC SUBPOPULATIONS IN VITRO

PHD DISSERTATION

by

Qiuyue Peng

AALBORG UNIVERSITY
DENMARK

Regenerative Medicine Group

Department of Health Science and Technology

The Faculty of Medicine, Aalborg University

Dissertation submitted: March, 2022

PhD supervisor: Prof. Vladimir Zachar,
Aalborg University

PhD committee: Associate Professor Tue Bjerg Bennike (chair)
Aalborg University, Denmark

Professor Emeritus Jens Høiriis Nielsen
University of Copenhagen, Denmark

Professor Susanna Miettinen
Tampere University, Finland

PhD Series: Faculty of Medicine, Aalborg University

Department: Department of Health Science and Technology

ISSN (online): 2246-1302
ISBN (online): 978-87-7573-923-3

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Stibo Complete, 2022

Qiuyue Peng

Date of birth: 07 August 1992

Telephone: +45 52826607

Education

2017 – 2022 Ph.D., Aalborg University, Denmark

2014 - 2017 Cand. Med., Zhengzhou University, China

2009 - 2014 BSc. Med., Henan University Minsheng College, China

Teaching and Scientific activities

2022	Lectures for pathology, 4th semester
	Lab course on 4th semester
2018 - 2022	Project supervision on 4, 9, 10 semester, and international exchange student
2021	Poster presentation: Immunophenotyping Profiling of Human Adipose-Derived Stem Cells During Expansion in Vitro, PhD day, Aalborg University
2021	Abstract: Analysis of Culture-induced Immunophenotypical Changes of Ex Vivo Human Adipose-derived stem cells (ASCs) in Multichromatic Flow Cytometry Data. 1st International Electronic Conference on Biomedicine (Online)

Publications

Peng, Q., Alipour, H., Porsborg, S., Fink, T., & Zachar, V. (2020). Evolution of ASC Immunophenotypical Subsets during Expansion in Vitro. *International Journal of Molecular Sciences*, 21(4), 1408.

Liu, L., Yu, Y., **Peng, Q**., Porsborg, S. R., Nielsen, F. M., Jørgensen, A., ... & Zachar, V. (2020). Distribution of Stromal Cell Subsets in Cultures from Distinct Ocular Surface Compartments. *Journal of ophthalmic & vision research*, 15(4), 493.

Peng, Q., Duda, M., Ren, G., Xuan, Z., Pennisi, C. P., Porsborg, S. R., ... & Zachar, V. (2021). Multiplex Analysis of Adipose-Derived Stem Cell (ASC) Immunophenotype Adaption to In Vitro Expansion. *Cells*, 10(2), 218.

Ren, G., **Peng, Q**., Fink, T., & Zachar, V., Porsborg, S. Potency Assays for Human Adipose-derived Stem Cells as a Medicinal Product towards Wound Healing. *Stem cell research & therapy*. Submitted. **Co-first author.**

Peng, Q., Ren, G ., Xuan, Z., Duda, M., Pennisi, C. P., Porsborg, S. R., ... & Zachar, V. Distinct dominant lineage from in vitro expanded adipose-derived stem cells (ASCs) exhibits enhanced wound healing properties. *cells*. Submitted.

ACKNOWLEDGEMENTS

The thesis: The immunophenotypical and functional characterization of ASC subpopulation *in vitro*, has been submitted to the Faculty of Medicine, Aalborg University, Denmark. This work was performed in the regenerative medicine, Biomedicine Group, Department of Health Science and Technology, Aalborg University, Denmark from 2017 to 2022. It is impossible for me alone to finish the project without warm help from the amazing people I ever met and collaborated with during this period. So, I would like to sincerely express my gratitude to:

First of all, Vladimir Zachar, my lovely supervisor. It is he who opens the biomedical door for me and lets me understand what is real science about. When I encounter challenges and difficulties, he is always there to support me unconditionally. From the perspective of others, he may appear to be "strict", but to me, he is just so highly demanding and hopes that I can become better and better. Such kind of interactive works quite well for me, and it can always motivate me to improve myself. Furthermore, I appreciate how quickly he revises my paper, allowing us to submit the paper on time. My heartfelt thanks go to him.

I would also like to thank Assoc. Prof. Trine Fink and Simone Riis Porsborg for supporting and inspiring me all the time. Whenever I need help, they are always there to guide me on how to solve the problems one by one. Their great solutions and kind supports are the power for me to conquer every challenge.

Lei Liu, the international medical manager at Novo Nordisk, is highly acknowledged. Without him, I cannot even be in Denmark, let alone get a Ph.D. degree. He is so smart, kind-hearted, patient, and reliable. I even cannot remember how many times I have been bothering him to ask for suggestions. And he can always come up with some practical and useful suggestions that make my project and life much easier.

Laboratory technician Lisa Engen is also acknowledged for her endless assistance, such as the purchase of reagents and technical guidance. Her sweet smile is like the sun in winter and makes the surrounding people feel warmer, especially in the cold and windy Aalborg.

Assoc. Prof. Cristian Pablo Pennisi, Hiva Alipour, and Fereshteh Dardmeh are acknowledged for their warm hands.

I would also like to thank my lovely office mates, Ph.D. Guoqiang Ren with his wife Jianfang Pu, and Ph.D. Zongzhe Xuan. When I am sad or frustrated, they always cheer me up by feeding me some wonderful Chinese food. I never feel alone with their companions.

The China Scholarship Council and S.C. Van Fonden are acknowledged for the financial support of my stay in Denmark.

In the end, I give my deepest gratitude to my parents, my big brother, and my sister for always spoiling me. As the youngest child, everyone always gives me the most of their care and love. Besides, my best friend, Yue Hu, is wonderful, too. We have known each other since we are high school and now we are still together in Aalborg. Thanks for her endless help, encouragement, and delicious food. In particular, I would like to give a big hug to my beloved boyfriend, Michael Sloth Bennetsen. Thanks for his unconditional understanding, thanks for driving from Kolding to Aalborg every weekend, thanks for his tolerance of my bad temper, thanks for massage, and thanks for all he has done for me. I should stop here, otherwise, I cannot help crying….

ENGLISH SUMMARY

Adipose-derived stem cells (ASCs) represent a heterogeneous mixture containing stem and progenitor cells in various stages of differentiation, whose ratio and properties also evolve along with *in vitro* expansion. Consequently, the overall biological effect of a given ASC preparation reflects the particular cellular composition, which currently is not possible to effectively control. ASCs can secrete a considerable number of biological molecules and hold an array of functions, and it is assumed that these properties are not equally distributed but rather associated with discrete subsets. Indeed, available evidence indicates that ideal regenerative medicine applications may benefit from the usage of a single clonal population derived from primary cells rather than the original complex mixture.

Therefore, the main aim of this thesis is to identify and categorize stem cell clonal lineage based on a combination of selected cell surface markers as well as to determine the immunophenotypical subpopulation with the most prominent functionality. Multi-color flow cytometry analysis or fluorescence-activated cell sorting technique will be employed to unveil the phenotypical repertoire and further enrich the subpopulations of interest. Proliferation rate, stemness properties (colony formation capacity, tri-lineage differentiation), and wound-healing related assay (scratch assay, tube formation assay) will be the main analysis to compare the functional difference between those targeted ASC subsets.

In Study I, 15 surface markers, combined in triplicates, were selected to investigate the individual expression level and the dynamics of co-expression variants along with *in vitro* culturing. The results confirmed a homogenization process taking place at passage 2 due to the exchange, convergence, or divergence of the minor markers, irrespective of donors. However, donor-dependent variation regarding the evolvement of ASC subpopulations exists.

In Study II, to more precisely define the ASC immunophenotypical subsets, two comprehensive flow cytometry analyses based on the co-expression of 7- or 5-fold multiplexing were established. After adaption to *in vitro* expansion, finite numbers of cellular subsets were discerned. Dominant clones were identified across all of the donors; however, the minor subsets were highly swing and donor-determined.

In Study III, while identifying these immunophenotypical subsets is critical for characterizing cell products in clinical settings, the potentials of each well-defined subpopulation cannot be fully defined solely by the phenotypical repertoire. Therefore, we purified two highly-defined ASC immunophenotypical populations for an array of tests and found that cells featured by $CD274^{+}CD146^{+}CD34^{-}CD36^{-}CD200^{-}CD248^{-}CD271^{-}$Stro-$1^{-}$ had a stronger colony-formation capacity and adipo-/osteogenic potentials as well as a higher efficacy in promoting wound healing.

In conclusion, these findings provide a better overview of the phenotypical characteristics of ASC entailing specific potentials and thus supply more information on ASC cellular subsets in personalized intervention.

DANSK RESUME

Stamceller fra fedtvæv (ASC'er) består af en heterogen blanding af celler indeholdende forskellige subpopulationer af stam- og progenitorceller på forskellige stadier af differentiering. Den præcise komposition af blandingen og dennes egenskaber udvikler sig i takt med at cellerne dyrkes *in vitro*. Den biologiske effekt af et givet ASC-præparat afhænger derfor af dennes cellulære sammensætning, som i øjeblikket ikke er mulig at kontrollere. ASC'er kan udskille mange signalstoffer og udføre forskellige funktioner, og det antages, at disse ikke er ens for de forskellige subpopulationer, men snarere forbundet til de individuelle celletyper. Faktisk indikerer tilgængelig evidens, at forskellige kliniske indikationer kan drage fordel af bare en enkelt subpopulation i stedet for den oprindelige heterogene blanding.

Derfor er hovedformålet med denne afhandling at identificere og kategorisere disse immunofænotypiske subpopulationer baseret på en kombination af udvalgte celleoverflademarkører undersøgt ved hjælp af multikromatisk flowcytometri. Ved at anvende fluorescensaktiveret celle-sorteringsteknik kunne subpopulationerne isoleres og subpopulationer med den mest fremtrædende funktionalitet identificeres. De centrale parametre til at sammenligne funktionaliteten af subpopulationerne var proliferationshastighed, stamcelleegenskaber (kolonidannelseskapacitet, differentiering) og sårhelingsrelaterede egenskaber (scratch assay, tube formation assay).

I studie I blev 15 overflademarkører nøje udvalgt og cellernes individuelle ekspressionsniveau af disse kombineret i triplikater undersøgt. Det blev også undersøgt hvordan dynamikken af co-ekspressionsvarianter ændrede sig under dyrkning af cellerne *in vitro*. Resultaterne bekræftede en homogeniseringsproces, der fandt sted gennem udveksling, konvergens eller divergering af de mindre markører. Disse tendenser var uafhængige af valget af donor. Der eksisterer dog donorafhængig variation med hensyn til udviklingen af ASC-subpopulationer.

I studie II blev der etableret to mere omfattende flowcytometri-analyser baseret på co-ekspressionen af 7- eller 5- markører for mere præcist at definerer de ASC-immunofenotypiske subpopulationer. Efter cellerne var dyrket *in vitro* og populationer havde fået lov til at tilpasse sig, blev det egentlige antal af subpopulationer kortlagt. Dominante subpopulationer blev identificeret for alle donorerne; dog var de mindre subpopulationer meget svingende i størrelse fra donor til donor.

I studie III blev to meget fremtrædende subpopulationer sorteret fra og undersøgt. For selvom at identifikationen af disse immunofænotypiske subpopulationer er vigtig for karakteriseringen af celleproduktet i et klinisk perspektiv, så kan potentialet af disse subpopulationer ikke beskrives blot ud fra deres udtryk af markører. Vi fandt her, at

celler der udtrykte CD274+CD146+CD34-CD36-CD200-CD248-CD271-Stro-1- havde en større evne til at danne kolonier, differentierer til fedt og knogle celler, samt var bedre til at fremme sårheling.

Som resultat af ovenstående har vi nu et bedre overblik over de fænotypiske karakteristika af ASCer, hvordan disse påvirkes af dyrkningen og deres specifikke potentialer. Alt dette er vigtig information for at gøre den ASC-baserede behandling mere målrettet til patienternes personlige behov.

LIST OF ABBREVIATIONS

ASC	Adipose-derived stem cells
α-MEM	Alpha minimum essential medium
BMI	Body mass index
BM-MSC	Bone marrow-derived stem cells
BSA	Bovine serum albumin
BV	Brilliant violet
BrdU	Bromodeoxyuridine
CD	Cluster of differentiation
CFU	Colony-forming unit
CV	Coefficient of variation
CCL20	Chemokine ligand20; macrophage inflammatory protein3
cGMP	Current good manufacturing practice
CXCL12	Stromal cell-derived factor1; C-X-C motif chemokine12
CPA	Cryoprotective agent
DMEM	Dulbecco's minimum essential medium
EPC	Endothelial progenitor cells
FACS	Fluorescence-activated cell sorting
FGF-2	Fibroblast growth factor
FMO	Fluorescence minus one
FVS570	Fixative viability stain 570
HDFs	Human dermal fibroblasts
HDMEC	Human dermal microvascular endothelial cell
HGF	Hepatocyte growth factor
HPL	Human platelet lysate
IL-4/6/10	Interleukins4, 6 and 10
IDO	Indoleamine 2,3-dioxygenase
IGF-1	Insulin-like growth factor 1
MFC	Multicolor flow cytometry
MFI	Median fluorescent intensity

MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
PGE2	Prostaglandin E2
PMT	Photomultiplier tube
PPAR-γ2	Peroxisome proliferator-activated receptor gamma2
PGE2	Prostaglandin E2
RUNX2	Runt-related transcription factor2
SA-ASCs	Supra adventitial adipose stromal/stem cells
STC1	Stanniocalcin1
S/N	Signal-to-noise ratio
SOX-9	SRY-box transcription factor 9
SP	Subpopulation
SVF	Stromal vascular fraction
VEGF	Vascular endothelial growth factor
Y-Med	Median fluorescent intensity of Y-axis
Y-Stdev	Standard deviation of Y-axis

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

1.1. ADIPOSE-DERIVED STEM CELLS

Mesenchymal stem cells(MSCs) are self-renewed cells that are able to differentiate into different specialized cell types of the tissue from which they are originated under certain physiological/experimental conditions[1]. MSCs were initially found and isolated from bone marrow and later from the placenta, umbilical cord blood, skeletal muscle, adipose tissue, liver, and intestines[2]. Despite the morphological, immunophenotypic, and functional similarities, bone marrow-derived (BM-MSCs) and adipose-derived stem cells (ASCs) are better well-known than other MSCs sources [3,4]. Both ASCs and BM-MSCs can recover the patients' defective tissue, but ASCs are more widely used [5–7], the reason for which is explained below.

ASCs have a lower sampling risk for donors and a relatively easier isolation procedure than BM-MSCs. Adipose tissue can generate a higher proportion of stem cells compared to bone marrow as the tissue source[7,8]. Growing researchers have highlighted the application of ASCs in regenerative medicine instead of embryonic stem cells due to a limited ethical issue. Besides, their immunoregulatory effects, anti-apoptosis, and anti-inflammatory potentials via bioactive secretion have been frequently investigated and reported[9–11]. *In vitro*, ASCs are characterized by plastic adhesion, colony-forming ability, rapid proliferation, and multi-lineage differentiation potentials[12–14]. Furthermore, these robust cells may undergo a long period of *in vitro* expansion without the loss of benefits and can easily survive from freezing[15].

Therefore, ASCs have been widely used for the treatment of chronic wound injury, fertility issues, hair loss, multiple sclerosis, and other conditions[16–18]. ASCs exhibit the greatest potentials for tissue repair and regulating the host inflammatory response after transplantation *in vivo* [10]. In addition, their immunosuppressive properties can greatly develop the efficiency of adipose tissue graft, because there is no need to consider human leukocyte antigen match between the donor and host[19]. Figure 1 illustrates the mechanisms underlying the ASC application in regenerative medicine as well as their benefits. Currently, more than 150 clinical trials (clinicaltrials.gov) are being conducted to validate the therapeutic effects of ASC-based intervention on inflammatory diseases, hepatic failure, orthopedic disorders, and autoimmune diseases (just list several examples here).

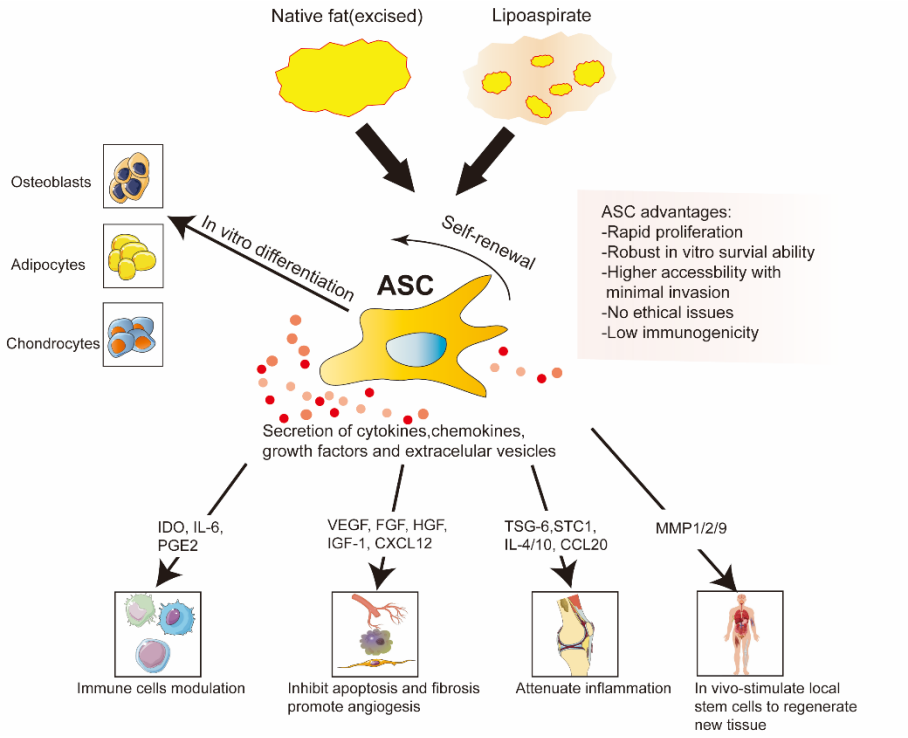


Figure 1. ASCs properties and advantages. ASCs are isolated either from excised fat or lipoaspirates. ASCs hold beneficial roles in regenerative medicine via secreting various trophic factors, maintaining differentiation capacity towards mesodermal, or stimulating local stem cells to develop new tissue, etc. Besides, ASCs are superior to other MSC sources. The curved arrow represents the self-renew ability. Abbreviations: ASCs, adipose-derived stem cells; CCL20, macrophage inflammatory protein-3; CXCL12, stromal cell-derived factor 1; FGF-2, basic fibroblast growth factor; HGF, hepatocyte growth factor; IDO, indoleamine 2,3-dioxygenase; IL-4/6/10, interleukins 4, 6 and 10; IGF-1, insulin-like growth factor 1; MMP1/2/9, matrix metalloproteinase-1/2/9; MSCs, mesenchymal stem cells; PGE2, prostaglandin E2; STC1, stanniocalcin 1; TSG-6, tumor necrosis factor inducible gene 6 protein; VEGF, vascular endothelial growth factor.

1.2. ASC PERIVASCULAR LOCATION

Adipose tissue gives rise to mesodermal. It has traditionally functioned as a metabolic reservoir to store and release energy substrates such as triglycerides, cholesterol, and fat-soluble vitamins. This concept was later modified in light of the dominant roles in sexual physiology played by sexual steroids[20]. Moreover, adipose tissues maintain thermal homeostasis and visceral statics while increasing rigidity and tissue resistances. Adipose tissue is essentially a collection of lipids that are filled with adipocytes, with the cytoplasm containing approximately 90% liquid[21]. Apart from stem/progenitor cells, other cell types, such as fibroblasts, pre-adipocytes, endothelial

cells, smooth vascular muscle cells, and resident blood cells, have previously been identified [22]. As a result, it is concluded that adipose tissue is made up of diverse cell populations.

Clear knowledge of the precise location of ASC within adipose tissue *in vivo* will improve the efficacy of purification and serve to more accurately identify progenitor cells that may originate from different tissues but exhibit similar multi-lineage differentiation capacity under the same microenvironment. Some researchers suggested that ASCs have a perivascular origin, where they co-exist with endothelial cells and pericytes. Others have demonstrated ASCs to be either pericytes or vascular stem/progenitor cells at various stages of differentiation located within the lining around the vasculature[23]. Blood vessels may also harbor (mesenchymal) stem/progenitor cells in their perivascular niche[24]. Here, we conduct a simple summary of previous studies that have revealed the ASC perivascular location *in vivo*.

ASCs($CD34^+/CD31^-/CD140\beta^-/\alpha$-smooth muscle action$^-$) were considered to coexist in capillaries with endothelial cells and pericytes or to act as specific fibroblasts in adventitial of larger vessels[25]. Zimmerlin et al. found that perivascular stromal/stem cells are divided into two separate subsets: one layer of the innermost components of $CD146^+/CD23^-/CD34^-$ pericyte cells, and one layer of outer $CD146^-/CD31^-/CD34^+$ supra adventitial adipose stromal/stem cells(SA-ASCs)[26,27]. Traktuev and coworkers[28] verified that $CD34^+/CD31^-$ ASCs were primarily established in the walls of microvasculature by the use of immunohistochemistry and immunofluorescence techniques, and these cells can maintain vascular stabilization by interacting with endothelial cells. Correspondingly, a perivascular stromal/stem cell subset has also been discovered in capillary and adventitial cells around large blood vessels, due to their native presence of MSCs markers and *in vitro* multi-differentiation ability[24,29,30]. However, because there are no specific markers that can unequivocally identify undifferentiated ASCs in situ, the precise location of ASCs within native tissue remains unknown. Figure 2 depicts adipose tissue constituents and ASCs' perivascular location *in vivo*.

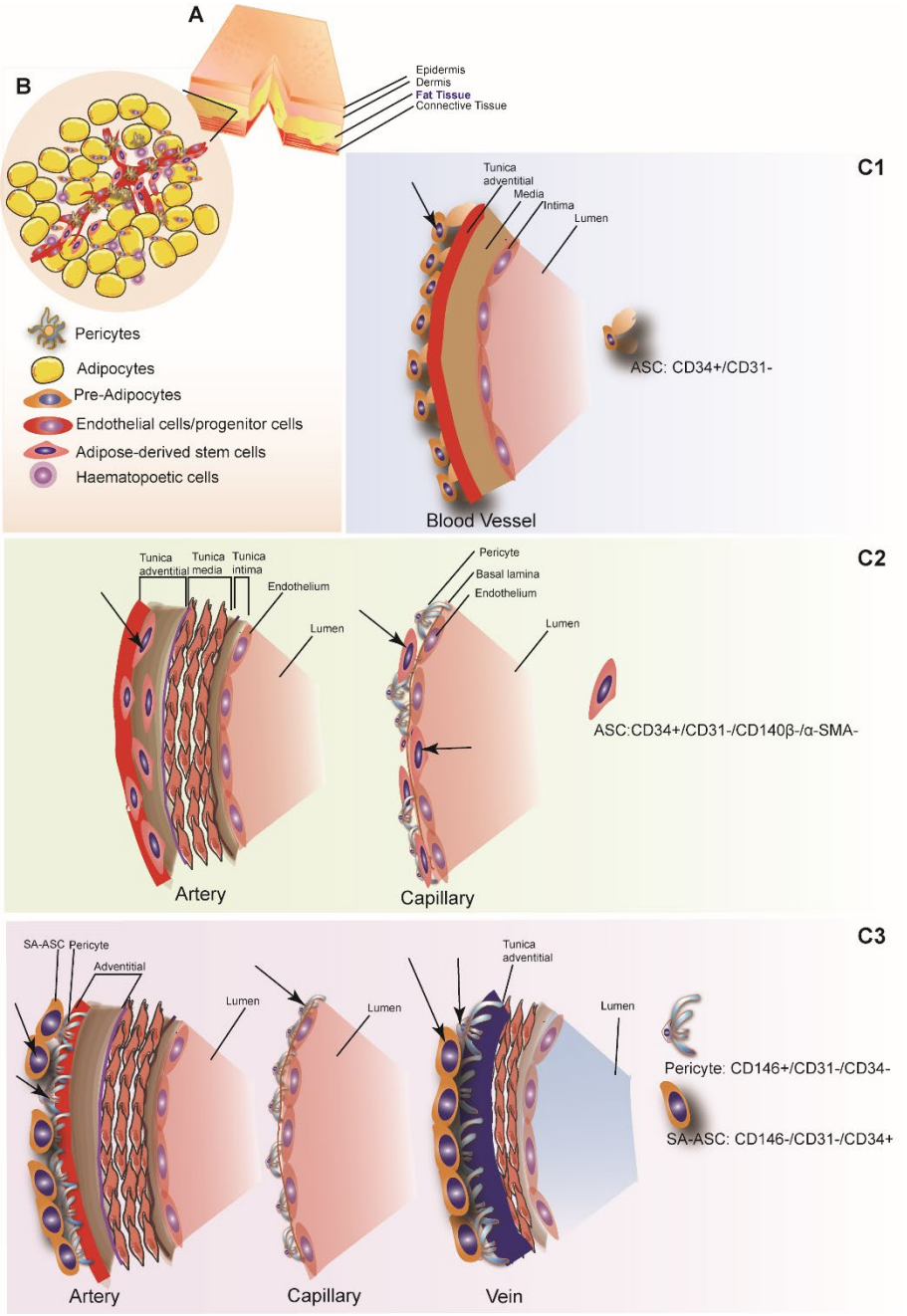


*Figure 2: ASCs resident in the adipose tissue. **A**: The origin of fat tissue. **B**: Cellular component of the fat tissue. **C**: The representative perivascular location of ASCs. Black arrows point to the location of ASCs in situ. **C1**: The location of ASCs*

(CD34$^+$/CD31$^-$) within adipose tissue occupy a pericytic position; ***C2****: ASCs (CD34$^+$/CD31$^-$/CD140β$^-$/α-SMA$^-$) exist as specialized fibroblasts in the adventitia of large vessels. In the capillary, ASCs coexist with their possible progenies, pericytes, and endothelial cells;* ***C3****: ASCs are organized into two distinct layers: pericytes (CD146$^+$/CD31$^-$/CD34$^-$), and SA-ASCs (CD146$^-$/CD31$^-$/CD34$^+$) in the larger blood vessels. Abbreviations: SA-ASCs, supra adventitial adipose stromal/stem cells; α-SMA, α-smooth muscle action.*

1.3. STROMAL VASCULAR FRACTION

Lipoaspirates or extracted fat (as previously mentioned) can be used to isolate stromal vascular fraction (SVF); the former adopts enzymatic digestion, while the latter needs the mechanical disruption of connective tissue. For both SVF preparation procedures, the removal of mature adipocytes with a high content of fat droplet (floating layer) will constitute SVF[31]. SVF is a heterogeneous cell mixture, and the cellular components are illustrated in Figure 3. Stem/progenitor cells account for approximately 3% of the total cell population [5,31–39]. Using flow cytometry, researchers attempt to identify distinct types of cells that differ in cell size, granularity, and expression level of surface molecules[40]. The characterization of each cell type and specific proportions of these constituents to each other are still under discussion. It was first reported that freshly isolated SVF contained a large number of CD34$^+$ cells, some of which featured CD34$^+$/CD31$^-$ cells with endothelial progenitor cells (EPC) properties[41]. Later, a more accurate SVF profile is revealed, as shown in Table 1. Yoshimura and co-workers have comprehensively characterized cell populations within SVF[39], along with the supplementary findings from other studies. Furthermore, some tried to determine the precise expression level of a single marker within SVF, whereas others focused particularly on the proportion of different cell populations[42–44].

Current flow cytometric data are insufficient for comparing the composition and efficacy of SVF, owing to a lack of specific unique markers for a clear definition. Sharing the common membrane antigens between different cell types makes it difficult to distinguish one cell type from the heterogeneous cell mixtures. Despite these ambiguous messages, SVF is still widely used in a variety of clinics. Andia et al. [45] described SVF formulations, mechanisms of action, and clinical application, focusing on musculoskeletal disorders, urogenital diseases, chronic wounds, cardiovascular and respiratory diseases, and chronic migraines. This review provides a better understanding of the biological effects of SVF in clinical practice.

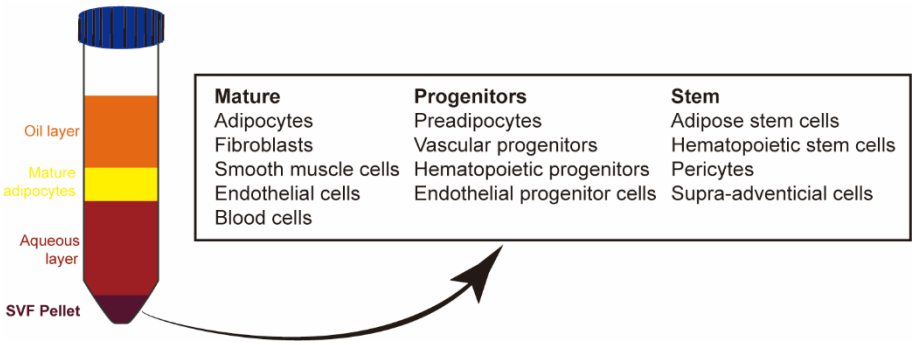


Figure 3 Cells inside SVF pellet. Abbreviations: SVF, stromal vascular fraction. The cellular compositions in the box are based on the work of Cohen and his colleagues[46].

Table 1. The constitutes of SVF

Cell type	[39]	[26]	[47]	[28]	[48]
ASCs	$CD31^-/CD34^+/CD45^-/CD90^+/CD105^-/CD146^-$		$CD90^+/CD29^+/CD34^-$	$CD34^+/CD31^-/CD4^-/CD144^-$	
EPC	$CD31^+/CD34^+/CD45^-/CD90^+/CD105^{low}/CD146^+$	$CD45^-/CD31^+/CD34^+$			$CD34^+/CD146^+/CD31^+$
Pericytes	$CD31^-/CD34^-/CD45^-/CD90^+/CD105^-/CD146^+$	$CD45^-/CD31^-/CD146^+$	$CD146^+/CD90^+/CD34^-$		$CD146^+/CD34^-/CD45^-/CD56^-$
Blood	$CD45^+$				
SA-ASCs	$CD45^-/CD31^-/CD146^-/CD34^+$	$CD45^-/CD31^-/CD14^-/CD34^+$		$CD34^+/CD45^-/CD31^-$	$CD34^+/CD31^-/CD146^-$
Single markers [42,43]	Nearly 11 % $CD14^+$, 2 % $CD31^+$, 7 % $CD34^+$, 9 % $CD45^+$, 29 % $CD90^+$ and 47 % $CD146^+$ 11 % $CD2^+$, 18 % $CD11a^+$, 29 % $CD14^+$, 49 % $CD31^+$, 57 % $CD45^+$ and 60 % $CD90^+$				
Proportion of subsets [44]	Hematopoietic-lineage cells: Endothelial cells 10-20% Stem and progenitor cells <0.1% Pericytes 3-5% Granulocytes 10-15% Stromal cells 15-30% Monocytes 5-15% Lymphocytes 10-15%				

Abbreviations: SA-ASCs, supra adventitial adipose stromal/stem cells; EPC, endothelial progenitor cells.

1.4. PHENOTYPE PROFILE OF *IN VITRO* CULTURED ASCS

With the continuous cultivation of SVF, the plastic adherent fraction is referred to ASCs. Studies have attempted to reveal a unique "CD signature" that could be assigned precisely to identify ASCs. Table 2 provides an overview of those key findings. There is commonality and disparity. CD9, CD10, CD13, CD29, CD34, CD44, CD49e, CD73, CD90, CD105, CD166, and the absence of CD11b, CD14, CD16, CD31, CD45, CD106, HLA-DR were frequently identified and agreed upon. However, disagreements exist: some researchers found CD34, CD54, CD107, and CD146 positivity, while others did not. In the case of CD34, a hematopoietic stem-cell-associated marker, up to 95% expression was detected after 7 days of culture, followed by a dramatic drop to no detection. Others, on the other hand, have only observed a minor CD34 loss during the cultivation process, and its expression could even be maintained for 10-20 weeks[39]. This reminds us to take the culturing window (a variable factor) into account because population doubling time affects the phenotypes, as illustrated by the examples (Table 2) of the dynamics of certain surface markers.

The donor, lipoaspirate procedure, isolation/expansion methods, and detection techniques may all contribute to the inconsistency of immunophenotypical repertoire. The characteristics of donors (age, BMI, ethnicity, and physical conditions) and lipoaspirate procedure (tissue site) vary between laboratories, resulting in a variation in the composition of the initially isolated SVF[49–53], which will be also described in the discussion section. Isolation/expansion methods, such as choice of enzyme, seeding density, basal media, supplements, coating reagents, antibody concentration, and oxygen addition, will affect cultured cells in their undifferentiated state as well. Our group examined the effects of the aforementioned factors on ASC characterizations[54]. Finally, multicolor flow cytometry (MFC), is a widely used method to detect the presence of surface epitopes; however, instruments setting, experimental setup, and cross-bleed compensation may exacerbate these inconsistencies as well.

Table 2: Surface expression profile of ASC

Positive marker	Negative marker	Ref.
CD9, CD10, CD13, CD29, CD34, CD44, CD49d, CD49e, CD54, CD55, CD59, CD105, CD146, CD166, HLA-ABC	CD11a, CD11b, CD11c, CD31, CD45, CD50, CD56, CD62e, HLA-DR	[55]
CD13, CD29, CD44, CD49d, CD71	CD14,CD16, CD31,CD34, CD45	[5]
CD13, CD29, CD34, CD44, CD49a, CD63, CD73, CD90, CD105, CD144, CD146, CD166, ABCG2		[56]
CD9, CD90, CD44, CD73.HLA-ABC	CD34, CD31, CD45, CD117 HLA-DR/DQ/ DP	[57]
CD13, CD29, CD34, CD36, CD73, CD90, CD105, CD166	CD11b, CD14, CD19, CD31, CD45, HLA-DR	[58]
CD13, CD29, CD90, CD105, HLA-ABC	CD14, CD19, CD34, CD45, HLA-DR	[59]
CD10, CD13, CD36, CD73, CD90, CD105	CD34, CD45, CD106	[44]
CD13, CD29, CD34,CD44,CD49d, CD73, CD90, CD105, CD166	CD45	[60]
CD73, CD90, CD105	CD31, CD34, CD45, SSEA-4	[61]
CD44, CD90, CD105, CD106, CD146, CD166, STRO-1, 3G5	CD14, CD31, CD45. CD106	[62]
CD29, CD49b, CD49d, CD49e, CD51, CD61, CD90, CD138, CD140a	CD11a, CD11b, CD11c, CD18, CD41a, CD49f, CD62L, CD62P, CD106, CD117, CD133, HLA-DR	[63]
CD29, CD34, CD36, CD73, CD90, CD105, CD146, CD166, CD200, CD201, CD248, CD271, CD274, Stro-1	CD31	[64]
Evolution trend	Up-regulated: CD13, CD29, CD44, CD63, CD73, CD90, CD166[56], CD44, CD49d, CD166[60], CD73,CD105,CD166, CD201[64]	
	Down-regulated: CD34,CD45 [60]	
	Fluctuate: CD34, CD146, CD271, CD20, CD248, CD274 [64]	
	Steady: CD29, CD90, CD36, Stro-1 [64]	

This table is edited based on the Zuk review[65].

1.5. ASC HETEROGENEITY AND POPULATION SELECTION

ASC culturing system represents a heterogeneous mixture, with fibroblast as a potential source of contamination due to their similar properties to ASCs, such as plastic adhesion, high proliferation rate (>50 population doubling before senescence), and immunoregulation capacity. Furthermore, ASCs in culture comprise real stem

cells and differentiation-committed progenitors that are at different stages regarding their proliferation rate, differential capacity, and senesce. Moreover, cells seeded at nonclone densities will produce a mixed adherent cell population, as some daughter cells may derive from clonogenic cells while others from nonclonogenic cells, resulting in a differential growth rate.

As mentioned in the introduction section, ASCs can release bioactive factors and thus conduct *in vivo/vitro* biological activities. It is unlikely that one clone (subtype) can perform all, therefore, it is plausible to infer that distinct clones are responsible for different functionalities within crude cell preparation. A better approach to clinical use in personalized medicine could be developed through a precise intervention of ASC subsets with a specific use. Many researchers are constantly working to unveil the specific applications of ASC subsets defined by surface epitopes. Clinical outcomes will undoubtedly benefit from a clear explanation of the link between immunophenotypes and functionalities. Therefore, we examined some previously reported and validated ASC immunophenotypical subpopulations with clear functionalities *in vivo/vitro*.

1.5.1. ASCS SUBPOPULATIONS *IN VITRO* STUDIES

CD73, CD90, and CD105 have been proposed by the International Society for Cellular Therapy as the positive surface markers for MSCs in an early-undifferentiated state. Some researchers have found a strong connection between ASC subsets featured by the three markers and enhanced functionalities. When myofibril and cardiac surface marker expression levels were compared, $CD73^+$ cells treated with 5-azacytidine had a higher cardiomyocyte differentiation capability than $CD73^-$ cells[66]. Rada et al.[67] discovered that $CD73^+$ cells exhibited a high chondrogenic but a low osteogenic differentiation ability, whereas $CD90^+$ cells had a high osteogenic differentiation potential. Chuang et al.[68] further confirmed the latter finding by demonstrating CD90 positive cells with the increased expression level of osteogenic genes as well as robust staining of alkaline phosphatase and alizarin red. Furthermore, $CD105^+$ ASCs, prepared by tissue-culture plastic, gel-embedded sheets[69], or biodegradable scaffold [70], displayed a greater chondrogenic ability.

CD271, a nerve growth factor receptor, is another promising subpopulation identifier. Murine $CD271^+$ cells can differentiate into adipocytes, osteocytes, and neurons *in vitro* at a significantly higher rate than $CD271^-$ cells[71]. Similarly, human $CD271^+$ cells were also found to have increased adipo-/osteogenic differentiation capacity, as well as a higher proliferation rate when compared to other unsorted cells and $CD34^+$ cells[72].

ASCs expressing CD34 *in vitro* are being extensively studied. $CD34^+$ cells exhibited a higher adipo-/osteogenic differentiation capacity than unsorted plastic adherent cells, as evidenced by Oil Red O and calcium content analysis, as well as a greater

proliferative ability[72]. The finding, however, contradicted what Suga and colleagues discovered[73], namely that the absence of CD34 conferred greater multilineage differentiation ability. These discoveries complicate the biological roles of CD34, motivating researchers to further conduct additional relevant studies in order to reach a clear conclusion. Doornaert et al. looked into the effects of co-culturing CD34 positive cells with mature adipocytes and confirmed that this co-culturing system resulted in rapid cell proliferation[74]. Furthermore, $CD34^+/CD90^+$ ASCs are gaining popularity, because they can differentiate into endothelial cells, resulting in a better capillary-like forming structure, and grow faster than the double negative cells, implying beneficial roles in tissue vascularization[75].

1.5.2. ASCS SUBPOPULATIONS *IN VIVO* STUDIES

Although *in vitro* functional studies can provide insight into subpopulation selection, the transition from tissue-culturing plastic to practical clinical application is imminent. The optimization of in *vivo* models is crucial to bridging the divide of laboratory-clinic scale.

When it comes to the subpopulations with MSCs hall markers expressed *in vivo*, CD90 enriched cells were found to have an enhanced osteogenic differentiation *in vitro* as well as increased bone formation when seeded on scaffolds for the repairmen of calvarial defects in mice models[68]. Meanwhile, $CD105^{low+}$ cells have been proposed to constitute a more osteogenic population, therefore, treatment with $CD105^{low+}$ cells differed significantly from treatment with other subpopulations (unsorted, $CD105^{high+}$, and scaffold only) on parietal bone defects [76]. Besides, $CD105^+$ enriched cells can integrate into host livers after being transplanted into mice to further improve basic liver function[77].

CD29, integrin β1, on the other hand, is well known as an MSC marker. Human CD29-enriched ASCs were thought to express more chondrogenic markers than $CD105^+$ cells after 6-week implantation in mice[78]. Furthermore, the CD271 subset ($Lin^-/CD271^+/Sca\text{-}1^+$) transplanted in conjunction with a biphasic calcium phosphate scaffold, has been used *in vivo* for osteoblasts' formation[79].

In one *in vivo* study, $CD34^+/CD31^-$ ASCs significantly improved blood flow and capillary density in ischemia mouse models[41]. Another study discovered that, after seeding $CD34^+/CD90^+$ cells in collage constructs and implanting them in mice for one month, clear tissue vascularization can be observed, whereas this vascular formation cannot be seen in the unsorted group[80], further validating the biological effects of $CD34^+/CD90^+$ cells *in vitro*. Besides, other groups reported the enhanced fat engraftment of $CD34^+CD146^+$ ASCs[81] and reduced chronic radiation-induced skin fibrosis after the intervention of $CD74^+$ ASCs[82].

CHAPTER 2. OVERVIEW OF PH.D. PROJECT

ASCs are widely used for numerous clinical trials, but there is a lack of evidence to demonstrate the potential therapeutic effects of ASC immunophenotypical subpopulations. Therefore, the main aim of this dissertation is to comprehensively investigate the phenotypical and functional characterization of highly defined ASC subpopulations after *in vitro* growth. To achieve this, we set two major objectives:

1. To explore the phenotypical characterization of possible ASC immunophenotypes throughout the *in vitro* expansion process.

2. To explore the functional characterization of those well-defined ASC immunophenotypical subsets.

In order to fulfill the aims mentioned above, we embarked on three studies, and indicated in Figure 4.

Study I: we evaluated the kinetic expression profiles of 15 selected surface markers at passages 1, 2, 4, 6, and 8 and investigated the variants of triple co-expression of molecules that were either MSC or ASC identification markers, or associated with immunoregulation, wound healing and differentiation.

Manuscript I: Evolution of ASC immunophenotypical subsets during expansion *in vitro*

Study II: we investigated the evolution of the highly defined cell subsets co-expressing with eight or five surface molecules at passages 1, 4, and 8, and highlighted the inter-donor variance.

Manuscript II: Multiplex analysis of adipose-derived stem cell (ASC) immunophenotype adaption to *in vitro* expansion.

Study III: we sorted two highly-defined ASC subpopulations ($CD274^{+}CD146^{-}$, $CD274^{+}CD146^{+}$, both negative for $CD34^{-}CD36^{-}CD200^{-}CD248^{-}CD271^{-}$Stro-$1^{-}$) for functional tests.

Manuscript III: Distinct dominant lineage from *in vitro* expanded adipose-derived stem cells (ASCs) exhibits enhanced wound healing properties.

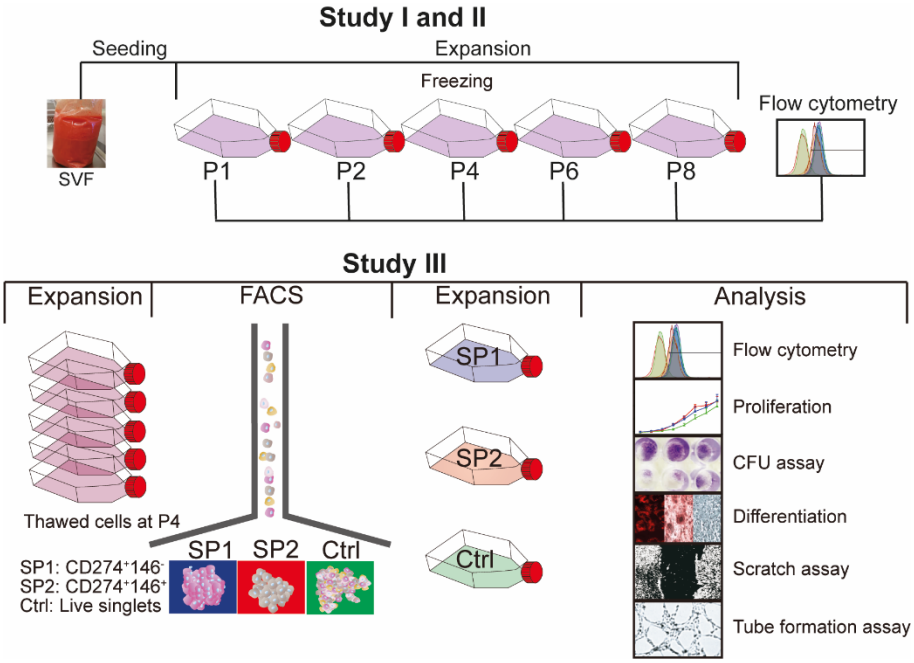


Fig 4. Experimental outline. Abbreviations: CFU: colony-forming unit; Ctrl: control; FACS: fluorescence-activated cell sorting; P: passage; SP: subpopulation; SVF: stromal vascular fraction.

CHAPTER 3. MATERIALS AND METHODS

The involved methodologies applied in the individual study are summarized in Table 3. The detailed procedure has been described in each manuscript. The choice of experimental methodology and materials in depth will be explained and discussed whenever found relevant.

Table 3: Methodology used in the individual study

Methodology	Study I	Study II	Study III
ASC isolation/expansion/cryopreservation	*	*	*
MFC	*	*	*
Fluorescence-activated cell sorting (FACS)			*
Proliferation, CFU, tri-lineage differentiation, scratch assay, tube formation assay			*

3.1. ASC PREPARATION

The adipose tissues used in these studies were obtained from donors who underwent cosmetic liposuction surgery at Aarhus University Hospital (Aarhus, Denmark) or Aleris-Hamlet Private Hospital (Aalborg, Denmark). After multiple steps listed in Figure 5, SVF suspension was collected and subsequently plated on tissue culture polystyrene.

When moving to the expansion stage, culture media, serum/alternatives, oxygen concentration, attach material, or seeding density may affect the cell properties[54], as referred the introduction section. Previous work in our laboratory has conducted a comparative analysis of media and supplements on the growth rate and surface markers[83], as well as research into the effects of hypoxia on ASC differentiation [84,85], wound-healing capacity[86], and long-term growth ability[87]. In this dissertation, we investigated, under a widely accepted standard culturing method, how the culture-related factors impacted the ASC subpopulations whose phenotypes were determined by the select surface markers. All cultures were ceased at passage 8 when cells can still optimally maintain their proliferative and stemness [88,89].

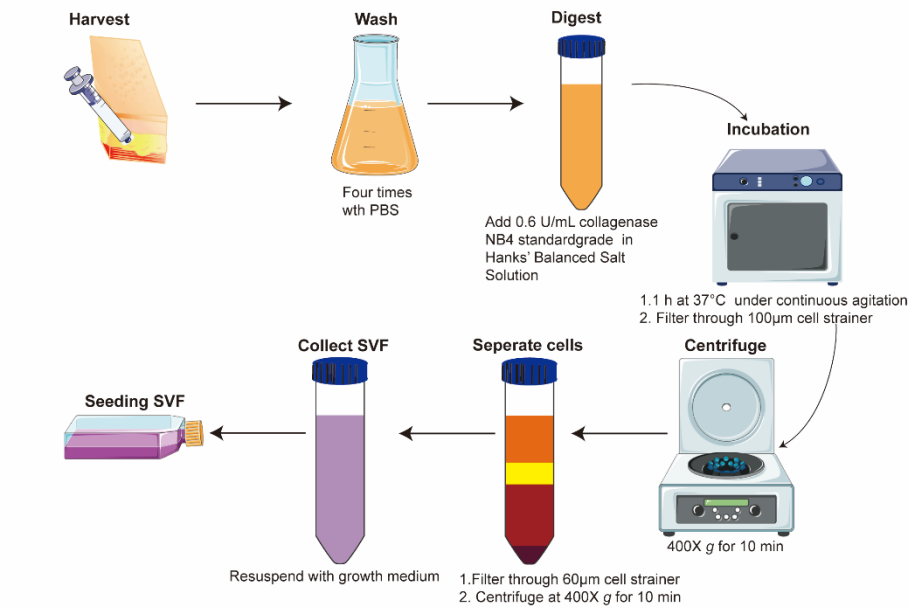


Figure 5. The isolation procedure for ASCs. Lipoaspirates were washed four times to remove the blood. After collagenase digestion and centrifugation, mature adipocytes with a high fat droplet can be separated as a floating layer, after the removal of which constitute SVF. The resulting SVF was then seeded in the culturing flasks.

3.2. FLOW CYTOMETRY

Surface epitope investigations conducted by MFC are a powerful tool for identifying and purifying ASCs, allowing to simultaneously measure multiple parameters. MFC frequently comes with some key technical challenges (Figure 6) that researchers must overcome in order to obtain satisfactory results, ranging from dyes selection, instrument setup to data interpretation. Accordingly, the following subsections compile a list of the key MFC technical aspects that are specifically relevant to our projects.

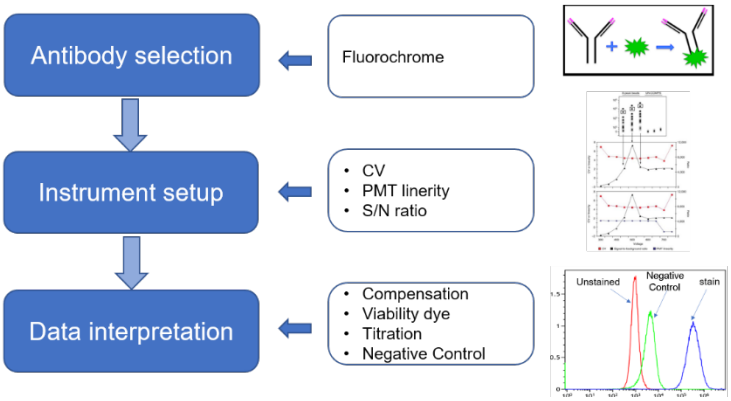


Figure 6. Key technique issue of flow cytometry. Abbreviations: CV, coefficient of variation; PMT, photomultiplier tube; S/N ratio, signal-to-noise ratio.

3.2.1. MFC

For ASC immunophenotype analysis, in Studies I/II, 15 markers (CD73, CD90, CD105, CD248, CD200, CD201, CD36, CD274, CD29, CD166, CD34, CD146, CD31, CD271, and Stro-1) were initially selected; either single marker expression level or the co-expression patterns were analyzed on 4-laser CytoFLEX (Beckman Coulter). CytoFLEX has a unique flow cell design equipped with integrated optics, innovative wavelength division multiplexing detection module, and avalanche photo-diode (APD), allowing for optimal light excitation/emission, reduced signal loss, and increased detection sensitivity for dim populations and nanoparticles[90]. Later, we discovered that ASCs were consistently positive for CD29 and negative for CD31, as shown in Manuscript I(Figure 2), therefore we excluded them from further studies. Accordingly, the remaining 13 markers were assigned to Study III and were analyzed using the MoFlo Astrios EQ (Beckman Coulter) equipped with four lasers including a UV laser. In Study III, MoFlo Astrios was employed because its equipped laser and more detector settings allow for a more comprehensive analysis of ASC phenotypical repertoire by combining more markers into one panel with a minimized resolution loss.

3.2.1.1 Panel design - choice of fluorochrome

To achieve the specific goals in each study, 15 (Study I/II) and 13 fluorescent antibodies (Study III) were assigned to different panels, a process known as panel design. The primary principle of panel design is to minimize the spillover between different detection channels while maximizing the data resolution. In our studies, triple markers were divided into 5 panels for Study I based on their shared functionalities, as well as two panels involving 7- or 5-multiplexing for Study II, and two panels containing 8-minor and 5-major markers for Study III. Notably, the classic mesenchymal marker group (CD73, CD90, CD105) was excluded in Study II in order

to avoid the repetitive work with the first manuscript. Tables 4-6 illustrate all the panels used in this dissertation.

Table 4. Panels in Study I

CytoFLEX	Band pass	Fluorophore	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5
405nm	450/45	BV421					CD201
	525/40	BV510	CD105				
	610/20	BV605		CD166			CD36
	660/20	BV650			CD29		
561nm	610/20	PE-CF594				CD146	
	585/42	FVS570	+	+	+	+	+
	780/60	PE-Cy7		CD271	CD200	CD34	
638nm	660/20	Alexa Fluor 647		CD248			Stro-1
	712/25	APC-R700			CD274		
	780/60	APC-Cy7				CD31	
488nm	525/40	FITC	CD73				
	690/50	PerCP-Cy5.5	CD90				

Abbreviations: FVS570, fixative viability stain 570; BV, brilliant violet. +, FVS570 was used in all the panels.

Table 5. Panels in Study II.

CytoFLEX	Band pass	Fluorophore	Panel 1	Panel 2
405nm	450/45	BV421	CD248	CD201
	525/40	BV510	CD200	
	610/20	BV605	CD166	CD36
	660/20	BV650		CD29
561nm	610/20	PE-CF594	CD146	
	585/42	FVS570	+	+
	780/60	PE-Cy7	CD34	
638nm	660/20	Alexa Fluor 647		Stro-1
	712/25	APC-R700	CD274	
	780/60	APC-Cy7		CD31
488nm	525/40	BB515	CD271	
	690/50	PerCP-Cy5.5		

Table 6. Panels in Study III.

MoFlo Astrios	Band pass	Fluorophore	Panel 1	Panel 2
355nm	395/25	BUV395	CD201	CD36
	525/50	BUV496		CD34
	740/40	BUV737		CD248
561nm	579/16	FVS570	+	+
	614/20	PE-CF594	CD105	CD274
	785/60	PE-Cy7		CD146
638nm	664/22	APC	CD166	Stro-1
488nm	513/26	FITC[a]/ BB515[b]	CD73	CD200
	710/45	Percp-Cy5.5[a] /BB700[b]	CD90	CD271

[a] fluorochrome-conjugated antibody in Panel 1; [b], fluorochrome-conjugated antibody in Panel 2.

3.2.1.2 Spillover source

The fluorochrome spectrum provides information on the exciting and emission spectra of one specific fluorophore, allowing investigators to choose the suitable exciting laser and detector filter to cover the optimal fluorescence signal. Spillover occurs when the fluorescence emission of one fluorochrome can be detected in a channel designed to measure the signal from a given fluorochrome[91], accordingly, it will mislead collected signals of the primary channel. The spectrum of fluorophore examines the overlap of emission wavelengths between each other in the same panel, and further advances to optimize the panel design. Appendix I depicts the spectrum of all the fluorochromes used in the same panel for Studies I-III.

There are three spillover sources: (1) adjacent detectors of one single laser, for example, the spillover of FTIC into PE detector; (2) residual base fluorescence of tandem dyes into another channel. Tandem dyes contain donor and acceptor fluorochromes. In most situations, the fluorescence of donor fluorochrome is efficiently transferred to acceptor fluorochrome via Forster- or Fluorescence-resonance Energy Transfer [92,93] however, there is always residual base emitting signal into other detectors; (3) cross-laser spillover. It occurs as a result of the tandem acceptor/donor's emission spectra being similar to that of another dye. Tables 7-15 examine the spillover source in each panel.

As expected, more fluorochromes in the panel, more spillover sources, thus a higher risk of loss of data resolution, which will be explained in the next sub-section. The choice of the number of antibodies introduced in a single panel is determined by the purpose of each experiment. In Study I, the primary goal was to investigate a relatively accurate expression level of each surface molecule, so we tried to minimize the analyzed number of fluorochromes to three to avoid resolution loss caused by spillover. As for the other two studies, we aimed to potentially explore distinct ASC subpopulations highly defined by the differential co-expression patterns; thus, the

number of fluorochromes was increased as many as possible for one panel, together with a generally acceptable control of data resolution.

Table 7: Spillover source in Panel 1 of Study I

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm		BV510					
488nm		**FITC**			PerCP-Cy5.5		
561nm							
638nm							

Cross-laser spillover because of similar emission spectra

The fluorochrome in bold denotes its spillover of emission wavelength into the other channel, which is also applied in Tables 8-15.

*Table 8: Spillover source in Panel 2 of Study I**

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm			BV605				
488nm							
561nm							PE-Cy7
638nm				Alexa Fluor 647			

**, No obvious spillover source is observed.*

Table 9: Spillover source in Panel 3 of Study I

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm				BV650			
488nm							
561nm							PE-Cy7
638nm						**APC-R700**	

Cross-laser spillover because of similar emission spectra

Table 10: Spillover source in Panel 4 of Study I

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm							
488nm							
561nm			PE-CF594				**PE-Cy7**
638nm							APC-Cy7

Residual spillover between tandems and their base

Table 11: Spillover source for Panel 5 of Study I

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm	BV421		**BV605**				
488nm							
561nm							
638nm				Alexa Fluor 647			

Residual spillover between tandems and their base

Table 12: Spillover source in Panel 1 of Study II

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm	BV421		**BV605**				
		BV510	BV605				
488nm		BB515					
561nm			PE- CF594				**PE-Cy7**
							PE-Cy7
638nm						**APC-R700**	

Cross-laser spillover because of similar emission spectra
Residual spillover between tandems and their base
Spillover into adjacent detectors of the same laser

Table 13: Spillover source in Panel 2 of Study II

Wavelength / laser	Fluorochromes						
	~450	~530	~610	~660	~685	~740	~780
405nm	BV421			**BV650**			
			BV605	**BV650**¤			
				BV650			
488nm							
561nm							
638nm				Alexa Fluor 647			
				Alexa Fluor 647			APC-Cy7

Cross-laser spillover because of similar emission spectra
Residual spillover between tandems and their base
Spillover into adjacent detectors of the same laser

¤, *These two dyes overlap each other.*

Table 14: Spillover source in Panel 1 of Study III

Wavelength / laser	Fluorochromes					
	~408	~550	~624	~675	~760	~815
355nm	BUV395					
488nm		FITC			Percp-Cy5.5 **Percp-Cy5.5**	
561nm			**PE-CF594**			
638nm				Alexa Fluor 647		

Cross-laser spillover because of similar emission spectra

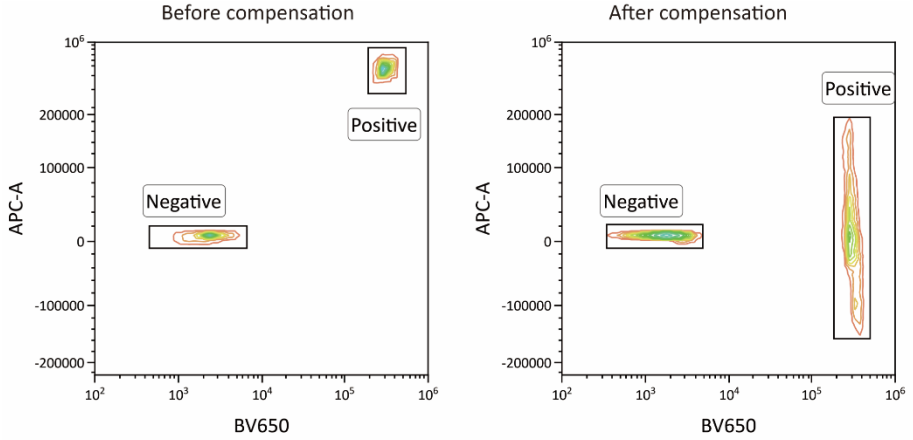
Table 15: Spillover source in Panel 2 of Study III

Wavelength / laser	Fluorochromes					
	~408	~550	~624	~675	~760	~815
355nm	BUV395	**BUV496**			**BUV737**	
		BUV496			BUV737	
488nm		**BB515**			**BB700**	
					BB700	
					BB700	
561nm			**PE-CF594**			**PE-Cy7**
			PE-CF594			**PE-Cy7**
638nm				APC		

Cross-laser spillover because of similar emission spectra
Residual spillover between tandems and their base

3.2.1.3 Compensation and data resolution

The compensation process in MFC entails mathematically removing spillover between applied fluorochromes. Matrix algebra is used to invert a spillover array to generate compensation values that are used to subtract out the contributions of non-primary fluorophores overlapping into a given channel[94,95] Compensation can remove the overlapped signals; however, this correction process will also increase the background of the negative population and promote signal spreading into other channels, causing difficulty to distinguish populations. Figure 7 exemplifies how the signals in BV650-channel influence APC-channel before and after compensation. As shown, although the spillover of BV650 into the APC detector is corrected by compensation (Y-median fluorescent intensity of the positive and negative population is proximately equivalent), the increased background of the BV650-negative signal and the spread of positive signal, which is quantitated by standard deviation, can still not be ignored or even amplified. This will attenuate the power to resolve various cell populations in the APC channel.



Gate	Y-Med	Y-Stdev
Negative	5,924.07	1,499.49
Positive	468,709.00	61,423.98

Gate	Y-Med	Y-Stdev
Negative	6,916.42	1,310.70
Positive	6,750.85	70,256.06

Figure 7. An example of fluorescence spillover introducing background/spread into another detector. Before compensation, an obvious spillover from the BV650 channel into the APC channel can be observed. After compensation, in the spillover detector (APC), the MFI of the positive population is roughly equivalent to that of the negative population, however, the spread (measured by standard deviation) caused by the spillover is not reduced and even amplified slightly. This will humble the resolution of any APC-positive cells from the negative population. Abbreviations: MFI, median fluorescent intensity; Y-Med, Median fluorescent intensity of Y-axis; Y-Stdev, standard deviation of Y-axis.

3.2.1.4 Practical experimental setting

As stated earlier (Figure 6), antibody titration, viability dye, or negative controls contribute greatly to the satisfactory results. A series of dilutions for each new antibody is used to determine an optimal point that separates the positivity from the background noise and acquires the best measurement of antigen expression levels; viability dye is applied to exclude the dead cells to remove nonspecific binding; fluorescence minus one (FMO) controls are utilized to define the positive and negative boundary. Notably, isotype controls with a long history are used to account for nonspecific staining of an antibody of a given isotype conjugated to a specific fluorophore. However, the background staining of each antibody conjugates varies due to the variation in specificity, concentration, aggregation degree, and fluorophore/antibody ratio of each antibody. Besides, isotype controls fail to take into consideration fluorescence spillover from other channels. These shortages significantly limit the use of isotype control in MFC.

3.2.2. FACS

3.2.2.1 Working principle

FACS technique can be used to capture and separate the desired cells with predefined features. As described in the introduction section, ASCs are constituted of heterogeneous cell clones, and FACS allows for a separation of targeted ASCs clones or populations for subsequent functional analysis. The general principle of FACS is the electrostatic deflection of charged droplets that contain particles of interest. Cells will be firstly labeled by fluorescence so that the operator can use computer software to define the fluorescent cells of interest when cells pass through the laser beam. By nozzle vibration, the downstream from the analysis point will be broken into discrete droplets so that the drops containing desired tagged cells will be charged and diverted from the mainstream into a collection receptacle while the remainders (uncharged droplets) are disposable into a waste container (Figure 8).

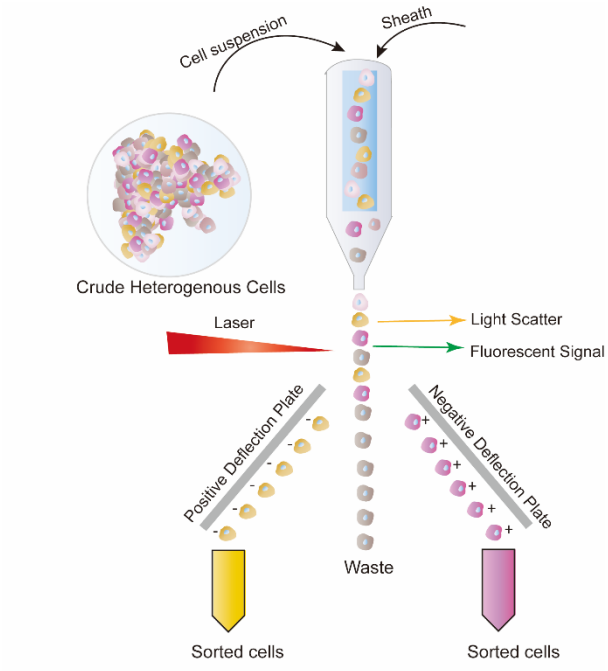


Figure 8. FACS working principle. The heterogeneous ASCs mixture is primarily stained by fluorescence-conjugated antibodies, and FACS will separate these fluorescence-labeled cells into different collection vessels. These drops containing desirable cells will be charged and diverted the mainstream into the collection tube, based on the operator's intentions.

3.2.2.2 Instrument setting (quality assurance)

A cytometer is equipped with laser beams (position and shape), filters (angels and quality), and photomultiplier tubes (PMTs, sensitivity, and resolution). A good flow system exhibits strong excitation of fluorochromes with good resolution and consistency over time, optimal transmission and reflection of emission light, and efficient signal collection at the detectors[96]. Laser efficiency and filter calibration are normally monitored by the technician from the supplier, thus, the optimization of PMT using a batch of calibration beads is discussed here.

Sphero Ultra Rainbow Six Peak Fluorescent Particles(Spherotech) were used to determine the lowest voltage range that yield the lowest coefficient of variation(CV) and highest signal-to-noise(S/N) ratio for each detector[97,98], the step of which was called PMT titration. The PMTs were further validated with the aid of BD compensation beads conjugated to our antibody, and the voltage point that provided the largest S/N ratio was selected. Besides, although the user manually calibrated the stream for the MoFlo Astrios EQ sorter, Sphero Ultra Rainbow Single Peak Fluorescent Particles (Spherotech) were still used to further optimize stream alignment and quality control along with the experiment.

3.2.2.3 Sample preparation

Superior cell preparation will improve sort purity, yield, post-sort cellular function, and viability. To maximize the overall quality of live cell sorting, the following factors are carefully considered: suspension buffer, single-cell preparation, dead cell discrimination, collection media, and cell concentration.

A good suspension buffer can maintain cell viability and prevent cell aggregates. As such, except for PBS and bovine serum albumin protein (BSA), non-phosphate system HEPES was supplied to maintain the neutral PH replicate incubator environment; proteolytic and collagenolytic enzyme-based Accumax solution was added to prevent the formation of clumping. As known, clumped cells will clog the sorting nozzle and impede sort performance. Except dissociation reagent added in the suspension buffer, cell samples were also filtered through nylon mesh before sorting. Dead cells will increase autofluorescence and non-specific binding, which will result in the loss of resolution. A good selection of viability dye will help to remove dead cells as well as maintain the post-cellular physiology for subsequent assays. Here, amine-reactive membrane-impermeable dye, BD Horizon Fixable viability Stain 570, was opted for dead cell discrimination. To improve recovery, the addition of protein in the sort collection media can reduce cell adherence to the tube wall, and the HEPES buffer in the collection buffer balances the drifted PH when cells travel from the nozzle to the collection receptacle. Besides, a pre-coat tube with collection media will also neutralize the plastic charge, which can reduce the droplet spray and tight adherence to the wall as well. A higher concentration of the cell sample will increase the

acquisition rate and shorten the sorting time so as to minimize the damage caused by sort-induced stress. On the other hand, a too high concentration of cells will greatly promote the formation of the aggregates, thus a range of $5x10^6$-$30x10^6$ per mL may be the appropriate concentration, which also depends on the cell type[99].

3.3. STEM CELL CHARACTERIZATION

The International Society for Cellular Therapy 2006 has proposed the minimal criteria for defining the multipotent MSCs: plastic adherence, positive or negative for specific surface molecules, and multipotent differentiation potential[100]. The plastic adherence was observed during the cell culture process, and the expression profile of surface markers was reported in Study I. therefore, in Study III, we targeted the differentiation potentials of those purified ASC subsets towards adipocytes, osteocytes, and chondrocytes to fully unveil their stem characteristics. For adipogenic differentiation, Nile Red was used to stain the formed lipid droplet, and the expression of peroxisome proliferator activated receptor gamma2(PPAR-γ2, differentiation-related gene) was detected to quantify the differentiation level. For osteogenic differentiation, Alizarin Red was opted to stain the calcium-containing mineralized matrix deposition and the gene expression of runt-related transcription factor 2(RUNX2) was assessed as well. For chondrogenic differentiation, alcian blue and dimethylmethylene blue was used to stain and measure glycosaminoglycans in cartilage; gene expression of SRY-box transcription factor 9(SOX-9) was quantified. Besides, the CFU assay was used to assess the frequency of progenitor cells.

3.4. PROLIFERATION CAPACITY

Apart from stem cell characterization, proliferation capacity is another indicator for evaluating the potential of these distinct subpopulations in future possible clinical applications, as a rapid proliferation rate will likely reduce the time required for sample preparation. ASCs are found a low proportion in adipose tissue, therefore, *in vitro* large-scale expansion is necessary to ensure the required number for repetitive therapeutic intervention until desired outcomes are achieved. There are currently four measurements for detecting cell proliferation: metabolic activity, cell proliferation markers, ATP concentration, and DNA synthesis[101]. To name a few examples, tetrazolium dye MTT and Alamar blue for detecting metabolic activity; Ki-67 as the proliferation marker; bioluminescent luciferase for measuring ATP concentration; bromodeoxyuridine (BrdU) assay for quantifying DNA contents. In our studies, we used Picogreen to measure the amount of double-stranded DNA from the homogenized lysates. Picogreen is a fluorescent dye that binds nuclear acid ultrasensitively, whose fluorescence can be detected by a spectrophotometer.

3.5. POTENTIALS IN WOUND HEALING

A large body of evidence has validated ASCs' prominent roles in promoting wound closure[102,103]. ASCs hold immunoregulatory properties that can accelerate the transition to a later stage during the inflammation stage, promote granulation formation during the proliferation phase, and speed up the deposition of the extracellular matrix during the remodeling phase. All of these effects listed above are merely brief summaries. Trevor et al. reviewed the detailed roles of ASC in each phase of wound healing[104]. According to this background information, the focus of our study is to look into of therapeutic effects of ASC-immunophenotypes on wound healing.

We used scratch assay to mimic the wound healing models in an attempt to investigate the re-epithelial effects of ASC-subsets derived condition media. The Wound Pin Tool was used to make a scratch on the monolayer of the human dermal fibroblasts (HDFs). One limitation of this assay is the inconsistency of scratch depth and size across wells. Besides, a manual procedure would be time-consuming and would result in a "ragged" edge boundary, leading to inaccurate data collection. Also, the biggest challenge, as Riis stated[105], is that this monolayer fails to comprehensively mimic the epidermis *in vivo*, which is composed of a multilayer of different cell types. There is a need to develop a 3D skin model. On the other hand, *in vitro* model is relatively inexpensive, simple, and allows for real-time measurement[106]. Furthermore, it will induce a robust directional migratory response, making data quantification quite simple[107].

We used tube formation assay to model the reorganization stage of angiogenesis, allowing us to assess the pro-angiogenesis effects of distinct ASC populations. The ability of human dermal microvascular endothelial cells (HDMECs) under the extracellular matrix support to form capillary-like structures was evaluated in terms of the junction/node numbers, length, and area of the formed tubes. One disadvantage is that HDMECs and extracellular matrix vary from lots to lots, making it difficult to obtain consistent, reproducible, and reliable data. This inconsistency was also visible in our preliminary experiments. We purchased Engelbreth-Holm-Swarm murine sarcoma ECM gel (Sigma-Aldrich) twice with different lot numbers, but capillary-like structures did not form in one type of gel, although both gels had the same catalog number. Additionally, *in vivo* studies should further be carried out to confirm the acquired results from *in vitro* assay, as commercially available endothelial cells have a competing proliferation rate, as well as heterospecific cell interactions that appear *in vivo* are absent. Despite these drawbacks, we should keep in mind that *in vitro* assay has a relatively easy procedure, requires a short period of intervention (typically 4-12 hours), and is compatible with high-throughput analysis. Notably, while Study III provides preliminary insights into the ASC subpopulation in wound healing, a full map of underlying mechanisms, such as immunoregulatory effects, merit further investigation, which is also our next goal.

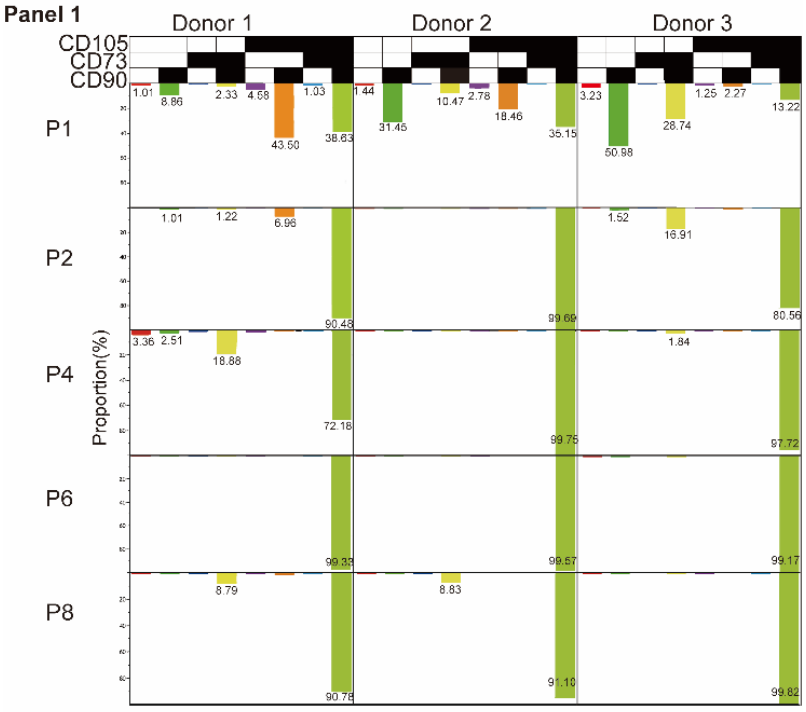
CHAPTER 4. SUMMARY OF RESULTS

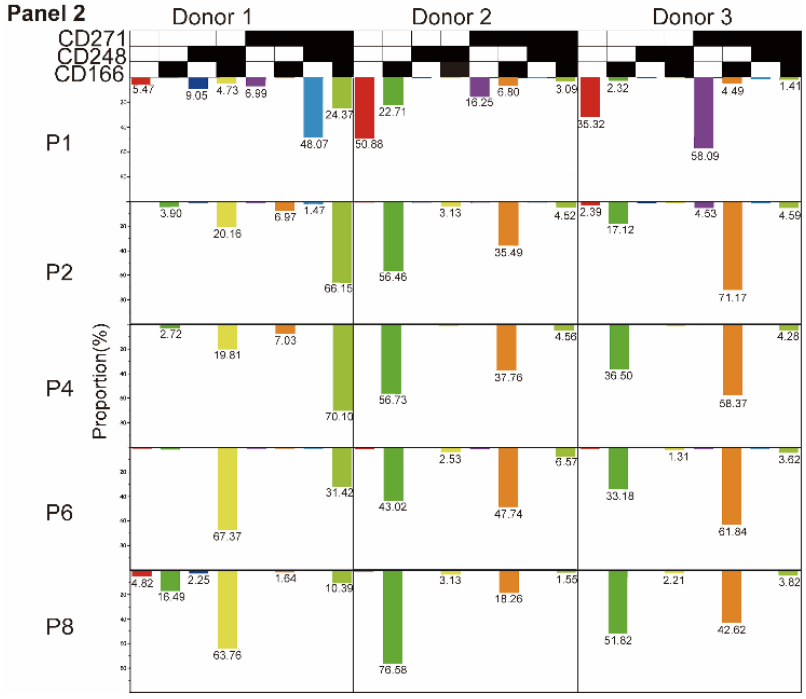
A detailed description of results in this dissertation has been described in each manuscript (see Appendix II). In this section, the main findings will be highlighted below.

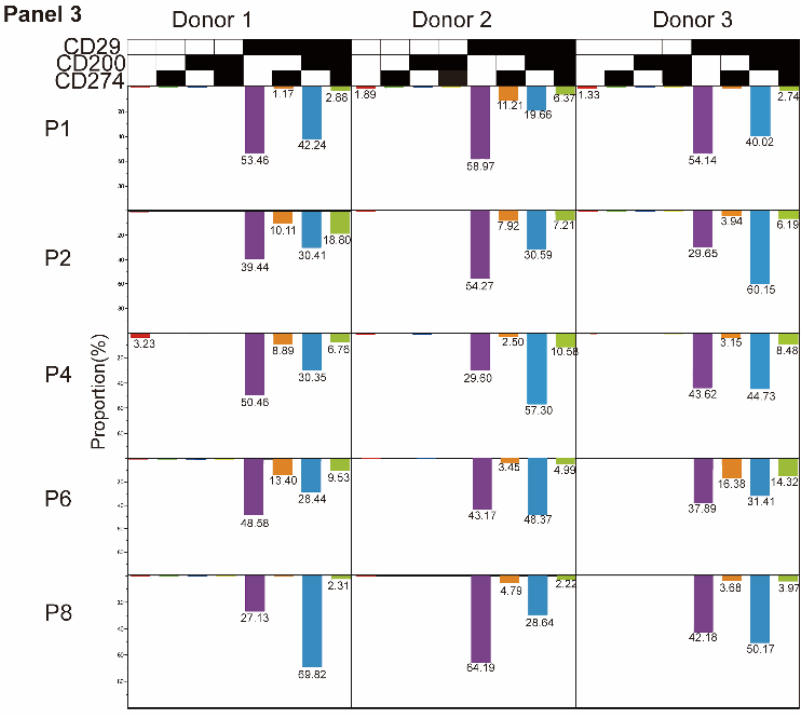
4.1. STUDIES I/II: CULTURE/DONOR-RELATED FACTORS ON ASC IMMUNOPHENOTYPICAL REPERTOIRE

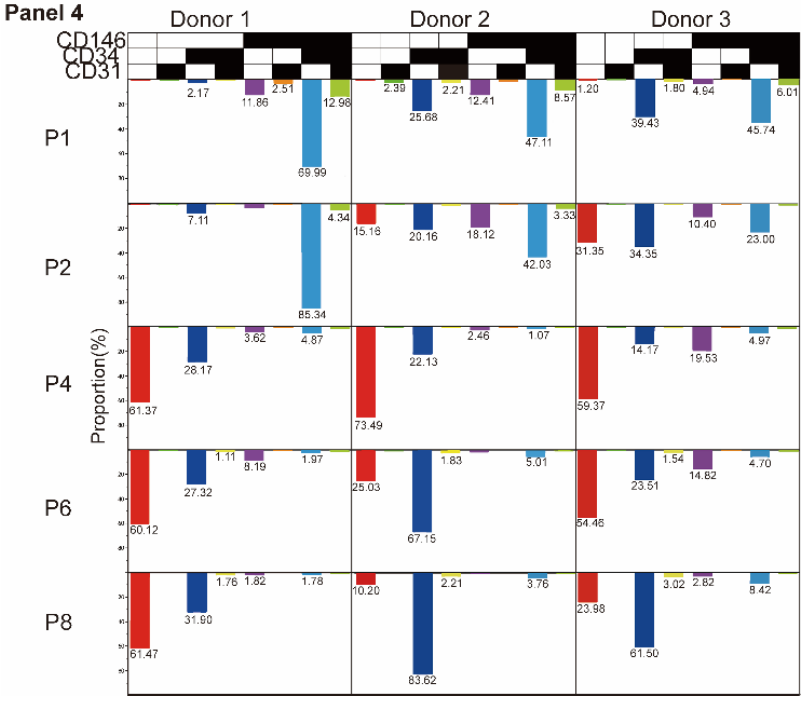
We employed MFC to evaluate the effects of culture/donor-related factors on the ASC immunophenotypes, as it allowed us to simultaneously analyze the level of single surface markers and co-expression of positivity and negativity. As for single surface expression profiling, throughout the whole observation process, ASCs were shown a presence of CD29, CD34, CD36, CD73, CD90, CD105, CD146, CD166, CD200, CD201, CD248, CD271, CD274, Stro-1, no matter it remained steady and fluctuated, and a gradual loss of CD31. Furthermore, three types of kinetic profiles for those 15 selected markers were observed: convergence to uniformity, continuous uniformity, and high fluctuation (Manuscript 1, Figure 2, and Figure 3).

When it came to ASC cellular subset compositions within the culturing system, a major homogenization process occurred due to a culture-related selection, which was demonstrated from 3-fold, 5-fold, 7-fold, or 8-fold multiplexing flow cytometry. Furthermore, only a limited combination of co-expression patterns was preserved at the later stage, which appeared and stabilized in a manner co-expressing with the major markers with CD73, CD90, CD105, CD166, CD29, or CD201, irrespective of the choice of donors (Figure 9, Figure 4 in Manuscript 1, Figure 2/3 in Manuscript 2). Such a homogenous procedure is because the minor subpopulations exchange, diverge, and converge into the dominant lineages, the underlying mechanism of which is validated in Manuscript 1, Figure 5.


Panel 1
Donor 1
Donor 2
Donor 3
CD105
CD73
CD90
P1
P2
P4
P6
P8
Proportion(%)
1.01 8.86 2.33 4.58 43.50 1.03 38.63
1.44 31.45 10.47 2.78 18.46 35.15
3.23 50.98 28.74 1.25 2.27 13.22
1.01 1.22 6.96 90.48
99.69
1.52 16.91 80.56
3.36 2.51 18.88 72.18
99.75
1.84 97.72
99.33
99.57
99.17
8.79
8.83
90.79
91.10
99.82


Panel 2
Donor 1
Donor 2
Donor 3
CD271
CD248
CD166
P1
P2
P4
P6
P8
Proportion(%)
5.47 9.05 4.73 6.99 48.07 24.37
50.88 22.71 16.25 6.80 3.09
35.32 2.32 58.09 4.49 1.41
3.90 20.16 6.97 1.47 66.15
3.13 35.49 4.52
2.39 17.12 4.53 71.17 4.59
2.72 19.81 7.03 70.10
56.73 37.76 4.56
36.50 58.37 4.28
31.42
43.02 2.53 47.74 6.57
67.37
33.18 1.31 61.84 3.62
4.82 16.49 2.25 63.76 1.64 10.39
76.58 3.13 18.26 1.55
51.82 2.21 42.62 3.82


Panel 3
Donor 1
Donor 2
Donor 3
CD29
CD200
CD274
P1
P2
P4
P6
P8
Proportion(%)
53.46
1.17
2.86
42.24
1.89
58.97
11.21
19.66
8.37
1.33
54.14
40.02
2.74
39.44
10.11
18.80
30.41
54.27
7.92
30.59
7.21
29.65
3.94
60.15
6.19
3.23
50.46
8.89
6.75
30.35
29.60
2.50
57.30
10.58
43.62
3.15
44.73
8.48
48.58
13.40
28.44
9.53
43.17
3.45
48.37
4.99
37.89
16.38
31.41
14.32
27.13
2.31
89.82
54.19
4.79
28.64
2.22
42.18
3.68
50.17
3.97


Panel 4
Donor 1
Donor 2
Donor 3
CD146
CD34
CD31
P1
P2
P4
P6
P8
Proportion(%)
2.17
11.86
2.51
12.98
69.99
2.39
25.68
2.21
12.41
47.11
8.57
1.20
39.43
1.80
4.94
45.74
6.01
7.11
4.34
85.34
15.16
20.16
18.12
42.03
3.33
31.35
34.35
10.40
23.00
61.37
28.17
3.62
4.87
73.49
22.13
2.46
1.07
59.37
14.17
19.53
4.97
60.12
27.32
1.11
9.19
1.97
25.03
67.15
1.83
5.01
54.46
23.51
1.54
14.82
4.70
61.47
31.90
1.76
1.82
1.78
10.20
83.62
2.21
3.76
23.98
61.50
3.02
2.82
8.42

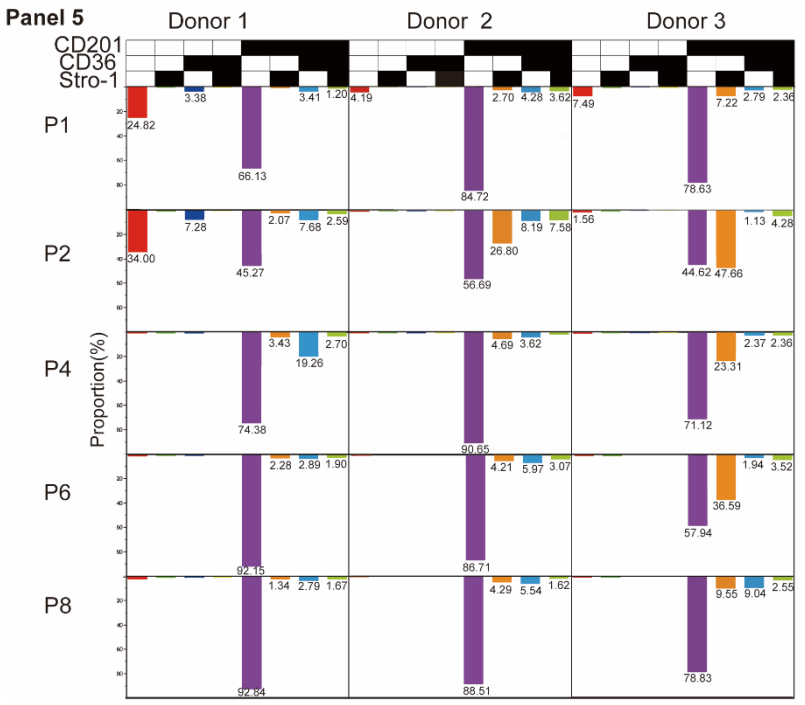


Figure 9. The proportions of each triple co-expression variant across each donor in Panels 1-5 and their dynamics over eight passages. The subsets whose proportions accounted for lower than 1% were not noted. Abbreviations: p, passage;

There is inter-donor variation in both cellular compositions and their evolution trend. Donor 1 in Study I differed the most from the other two, especially in Panel 2, as well as in Panels 4 and 5(Figure 9). In study II, throughout the whole analysis procedure, ASCs were dominated by several highly prevalent clones cross most of the donors, however, minor subsets were highly fluctuating and donor-dependent.

4.2. STUDY III: DISTINCT IMMUNOPHENOTYPES DISPLAYED DIFFERENTIAL FUNCTIONALITIES

Identifying the major subsets established on the combination of cell surface markers is critical to characterizing the cellular product potentially applied in clinics, however, immunophenotype alone cannot fully uncover the therapeutic potentials of the ASC subpopulations. Therefore, we carried out Study III to better visualize the ASC immunophenotypes and their associated functionalities that manifested during culture.

Upon *in vitro* expansion, two subpopulations, $CD274^{+}CD146^{-}$ (Subpopulation 1, SP1) and $CD274^{+}CD146^{+}$(Subpopulation 2, SP2), supplemented with $CD34^{-}CD36^{-}CD200^{-}CD248^{-}CD271^{-}Stro\text{-}1^{-}$, gradually appeared to outgrow than others. We decided to

further investigate these two clonal lineages. To catch up with the clinical procedure, where the frozen-thawed ASC products were applied, we opted to freeze the cells at passage 4 and examine the potential of targeted subpopulations after thawing. Strikingly, besides the two dominants described earlier, all the other detectable variants acquired the co-expression of CD248. After a period of propagation to ensure a sufficient cell number for functional assay, all the fractions shared similar immunophenotypical profiles, where $CD274^{+}CD146^{+}CD248^{+}$ and $CD274^{+}CD146^{+}$ harbored the highest proportion of cells. Collectively, all the sorted phenotypes after propagation cannot be maintained.

The functional analysis suggested that $CD274^{+}CD146^{+}$ cells outperformed $CD274^{+}CD146^{-}$ clones in terms of growth rate, clonogenic potentials, and capacity toward wound healing.

CHAPTER 5. DISCUSSION

The ASC-based products have been widely used for the treatment of various diseases in recent decades. The major challenge associated with the preparations of ASC for regenerative applications is that ASCs represent a heterogeneous mixture entailing undesirable therapeutical agents, which may dampen the expected outcomes. Augello et al. [108] emphasized that a single MSC clone can be highly heterogeneous, harboring the undifferentiated stem/progenitors or lineage-restricted precursors whose proliferation rate and differentiation capacity are varied. This compelled us to uncover the possible compositions of ASC subpopulations within crude ASC preparations, as well as the potential biological effects of well-defined immunophenotypical lineages. Such a study, we believe, will provide additional information for better targeting subpopulations of interest in personalized clinical settings.

5.1. FACTORS CONTRIBUTING TO ASC INCONSISTENCY

In Manuscript I, except for CD73, CD90, CD105, CD166, CD29, and CD31, other selected surface markers differed from the previous studies regarding both the expression level and their evolution trend. In Manuscripts I and II, donor-dependent discrepancies for quite a few ASC immunophenotypical subsets were also discerned. In addition, a direct donor-to-donor comparison in terms of their capacity to proliferate, differentiate, form new colonies, and heal the wound was conducted and identified as well in our group (data not published yet). Accordingly, an overview of the critical affecting factors is provided in Figure 10.

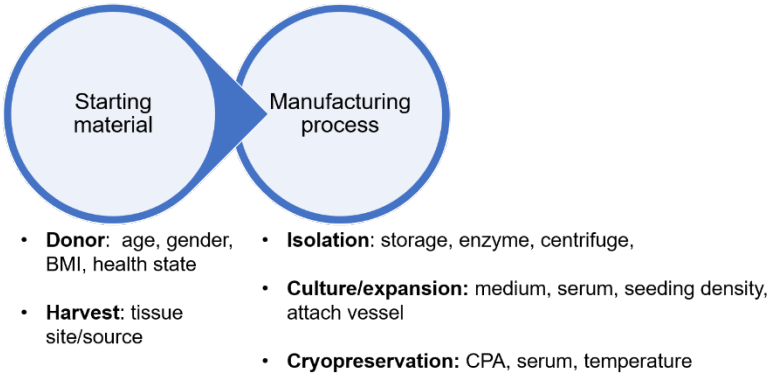


Figure 10: Critical factors leading to ASC inconsistency. Abbreviations: BMI, body mass index; CPA, Cryoprotective agent.

As for the starting origins where ASC are originated from, the donor's physical conditions, including age, gender, body mass index (BMI), or health state will be

taken into warrant consideration. Distinctiveness in ASC proliferative, differentiate, secretory, or antiapoptotic ability may be caused by differences in donor age/gender[109]. Take one example here, when compared to cells isolated from older subjects, infant-derived ASCs have a competitive advantage in terms of angiogenesis and osteogenesis formation[110,111]. Besides, increased BMI negatively correlated with the proliferation and differentiation potentials[112], although some studies failed to detect a significant relationship between BMI and ASC functionalities[113,114]. Concerning the donor's health state, such as chronic diseases, it will undoubtedly alter the ASCs' intrinsic properties or impair cell functions including stemness, differentiation, and angiogenic potentials[115–117]. When it comes to the harvest site, the abdomen has been described as the best origin, as superficial fat displays heightened multipotency and stemness features than the deep depot[113,118,119].

Manufacture procedures will affect the therapeutical effects of end cellular products, which are modulated by the choice of optimal cell isolation, culture/expansion, and cryopreservation method. Despite the fact that enzyme-digested SVF is already used in clinical trials[120,121], it is still necessary to acknowledge that enzymatic digestion-based protocols were originally applied for experimental purposes, but discovered to have relatively lower efficiency in clinical settings, due to unexpectedly higher cell death and antigens alteration[122]. Favorably, a new one-step and quickly-performed isolation method that does not require collagenase digestion can improve the quantity and quality of isolated stem cells[123]. Currently, the most commonly accepted medium for culture/expansion is Dulbecco's minimum essential medium (DMEM) or the alpha minimum essential medium (α-MEM), supplemented with 10% fetal bovine/calf serum[124]. However, more considerations still need to be taken into account to standardize the preparation procedure and obtain a high quality of ASC-based cellular product[125–128]. The addition of supplements or glucose, for example, can also change ASC functionalities[129,130]. Freshly-obtained tissue or cells are initially applied for most basic and clinical research, but it is approaching impossible to work on fresh material for all kinds of studies. This impetus a need to use the frozen-thawed cells stored using the cryopreservation technique, but give rise to additional disparities because of the distinct choice of cryopreservation media, time, temperature, and rounds. Increased cryopreservation time negatively impacted cell number and viability, but cell growth rate and stemness after recovery were maintained[89]. Furthermore, the beneficial effects of one type of Xeno-free cryopreservation media on ASCs plating efficiency, multipotency, or chromosomal normality were demonstrated as well[131,132]

Lastly but importantly, there are still methods to minimize the level of heterogeneity to some extent, leading to a relatively comparable comparison between different operators. A sequential wash procedure can purify ASC cellular compositions, as it will take different time-lapse for ASC clonal lineages to adhere to the culture surface. This phenomenon was also demonstrated in Studies I and II, where, upon *in vitro* expansion with continuous washing, the crude cell cultures became more

homogeneous. Another ensuing effort is to isolate the specific ASC immunophenotypical subpopulations by means of flow cytometric sorting or immunomagnetic techniques, which is also the aim of Study III.

5.2. SUBPOPULATION EVOLUTION AND POTENTIAL USE

As seen in Manuscripts I and II, only several possible combinations can be identified until the end of culture, and ASC cellular composition gradually acquired stabilization. Two major hypotheses for the mechanism underlying the temporal change: differences in proliferative capacity among distinct subpopulations; differential expression of epitopes. Based on our current results, it can only be inferred that the major driving force originates from the differential expression levels of the epitopes, especially those highly-expressed surface makers. Whether those subpopulations outgrow or proliferate faster than the others deserves a thorough exploration in which those targeted subpopulations are separated and measured individually.

In the introduction section, it is mentioned that immunophenotypical ASC subpopulations are advantageous for specific applications in regenerative medicine. However, there is still a long way to go before ASC subsets-based therapy can be used broader. Although the cellular compositions will more or less stabilize after a period of growth, it remains unclear whether those dominant ASCs subpopulations will change their potentials after manufacture but before the practical application, as suggested in Study III. This part of the content will be discussed in-depth in the following section. Furthermore, special consideration should be given to the minor subpopulations whose biological effects may be critical but challenging to identify or confirm. Firstly, they are highly susceptible to *in vitro* adherence to plastic, resulting in a fluctuating presence within the culturing, necessitating the optimal selection of passing time. Secondly, the current collection of targeted subpopulations is mainly based on either flow cytometric sorting or immunomagnetic technique (as indicated earlier), but the acquired cell number is far behind the demanding amount for practical clinical use. Alternatively, a further step, large-scale expansion, is required to produce sufficient numbers, however, the phenotype mutation and unknown alteration in ASC performance are likely to occur, making the situation more ambiguous.

5.3. FUNCTIONALITY UNDERLYING IMMUNOPHENOTYPICAL COMPOSITION

Currently, flow cytometrical analysis of ASC immunophenotype is a common method to identify the characterization of ASC and their subsets. We believe that each surface markers or their unique combination represent exclusive significance for cells. As a result, in Study III, we determined how the ASC co-expression subpopulations based on the negativity and positivity of specific epitopes behave during culturing, with the goal of identifying the specific link between immunophenotypical composition and

functionalities. So far, to our knowledge, previous studies have highlighted ASC immunophenotypes based on a single epitope or simple CD combination, after early isolation. However, it has not yet been determined how the functionalities underlying each immunophenotype persist or whether can be further regulated during culture.

We discovered that in Study III, it is nearly impossible for those collected subsets to maintain their immunophenotypical profiles the same as when they were sorted, but their functionalities continue to differ, despite a gradual approach to a similar phenotype. Specifically, the cellular composition of progeny cells from the sorted $CD274^+$ cells was organized similarly to that of $CD274^+$CD146+cells, both subsets being negative for $CD34^-CD36^-CD200^-CD248^-CD271^-$Stro-$1^-$, but the progeny from former culture failed to hold the endothelial and fibroblast support. We hypothesize that using the sorting technique, it is possible to purify genetically discernable populations of stem/progenitors or precursors which are functionally constitutive linking with those pre-defined phenotypes, and original transcriptional profiles from those purified subpopulations could be inherent to their progeny cells. As a result, the progeny cells differ in ways similar to when they are freshly sorted, although these daughter cells may share common cellular constitutions. This suggests that changes in phenotypical profiles exert negligible effects on the properties of the isolated cells, despite long-term *in vitro* propagation. However, the hypothesis requires a direct comparison of transcriptional profiles of those targeted cell populations, implying that a high-throughput RNA sequencing needs to be conducted on cells collected from both immediate-sort and progeny after propagation.

5.4. THE EFFECT OF FREEZING-THAWING PROCESS ON ASCS CHARACTERIZATION

As explained in the result section, to comply with the clinical procedures where large-scale frozen-thawed ASCs opted, we cryopreserved cells at passage 4 and examined the potentials of target subpopulations after thawing. Except for the SP2-like phenotype (double-positive for CD274 and CD146), the abundant cells acquired a co-expression of CD248 with an obvious deduction of the SP1-like phenotype (single positive for CD274). This indicates that the freezing-thawing process may alter the properties of ASCs subpopulations, at least on the phenotypical level. Indeed, some researchers have already concluded that cryopreservation can preserve the stemness of crude cells while resulting in acceptable viability and proliferation rate, but it can also cause changes for some surface markers[133–136]. However, there is a lack of evidence demonstrating how freezing affects subpopulation distributions and alters the representing functional characterization of individual lineages. Similar to ASC-based applications, a freezing procedure is crucial for storing the “off-the-shelf” ASC subpopulation; therefore, future research could be directed to the investigation of cryopreservation on the safety and efficacy of ASC subsets-based products.

5.5. PROLIFERATIVE, CFU, AND DIFFERENTIATION CAPACITY

In our studies, purified subsets demonstrated a more robust ability to proliferate, form colonies, and direct to adipo-/osteogenesis, compared to the crude mixed cell culture. It was inferred that heterogeneous cells were contaminated by other populations of more committed progenitors or precursors, dampening their stem potentials[137–139]. The enhanced capabilities of purified immunophenotypical subsets in Study III may facilitate their broader use. Take proliferation rate as an example, with a shorter doubling time, these clones (SP1 and SP2) will require significantly less time during preparation. However, it was noted that other studies discovered that $CD146^+$ cells had a longer doubling time than $CD146^-$ cells, contradicting our findings that CD146 positive and negative cells (SP1 and SP2 cells) showed comparable proliferation rates[140–142]. This discrepancy could be attributed to inconsistencies in phenotypic characteristics as well as a distinct MSC source. Concerning stemness, others reported that $CD146^-$ and $CD146^+$ did not differ in terms of CFU and tri-lineage differentiation under normal media, but when incubated in specific condition media, $CD146^-$ showed less osteochondrogenic differentiation than $CD146^+$ cells[140,141]. In our study, we also found that SP2 with the presence of CD146 differentiated the most, though there was no significance for chondrogenesis. Furthermore, our findings are consistent with previous research showing that $CD146^+$ cells have favorable adipogenic properties[27,142], demonstrating that these cells may have a greater potential to form more mature adipocytes after transplantation to a damaged area.

5.6. WOUND HEALING POTENTIAL

The supernatant from SP2-fracted cells positive for CD146 was found to improve the capillary-like formation of HDMECs and enhance HDF proliferation. Previous studies have linked CD146 positivity in MSCs to pro-angiogenic effects [143,144], the mechanisms of which are derived from their pericyte origin and secretory profiles[98,142]. The CD146 pericyte phenotypes may interact with endothelial cells to regulate vessel formation[145,146] and secrete vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin-1, all of which promoted vessel growth[81,142]. Such proangiogenic capacity of surrogate maker CD146 could partly explain why condition media harvested from SP2 cells ($CD274^+CD146^+$) behaved more advantageous in wound healing assays than others. Indeed, some researchers have already attributed the enhanced tissue formation and graft retention, after ASCs or ASC subset transplantation in animal models, to their robust vascularity[81,147,148]. However, there is still a long way to go between the preclinical study and the practical clinical application.

5.7. LIMITATIONS

The number of donors is the first concern. The minimum biological required number (n=3) in Studies I/II was used, as it is sufficient to demonstrate both the emergence of

dominant subpopulations and inter-donor variability. However, in order to fully map the range of variability and to determine the correlation of clinical data, a prospective study focus on the donor difference should be conducted. Furthermore, a direct comparison of ASC subsets enriched from donors categorized by different features should be conducted to fully uncover the effects of personalized information on the physiologies of ASC lineages.

In Study III, we highlighted the roles of CD274 and CD146 double-positive cells in the constitution of ASC clones. However, what are the roles of other well-studied markers in the combination system, such as CD271(differentiation-commitment)[149], or CD248(angiogenesis)[150], to name a few here? They did not appear to be involved in the combination of major discernible clones, as they distributed dispersed in many small variants. It is unclear whether they bear some specific functionalities, but any isolation of these small variants entailing these markers for functional assay will meet huge challenges. This is because *in vitro* expansion following sorting may introduce an unknown alteration within each subset as well as technical limitations.

Another consideration is about the models assessed for wound healing, as explained in the method section. *In vitro* models could be partly used to explore the potentials of these ASC lineages on wound healing, but they fail to mimic the practical wound process *in vivo* where multiple layers of cells or cell interactions are involved simultaneously.

Finally, MFC was applied throughout all the studies. When involved in the co-expression of 7-fold (Study II) and 8-fold (Study III) markers, the resolution loss caused by the compensation process is not likely to be ignored. As a result, it is quite difficult to resolve those minor lineages which distribute dispersedly within the culturing. Therefore, only finite numbers of combinations can be established when using MFC in Studies II/III, although there could be 2^7or 2^8 possibilities. It is believed that the panel designs for each study can still be optimized to achieve a better resolution.

CHAPTER 6. CONCLUSION

In conclusion, the studies provided a deep insight into ASC heterogeneity and the biological effects of those represented ASC immunophenotypes. We found that a major homogenization process occurred at passage 1 and several dominant ASC clones with co-expression of major markers were discerned and stabilized within crude culturing, regardless of donors. Besides, the inter-donor variance was present in terms of the cellular composition and the evolution trend of ASC clones. Furthermore, purified ASC immunophenotypical lineages can be used for specific interventions, whose functionalities appear determined at the sorting point and retained inside cells, irrespective of later changes in immunotype repertoire. Surrogate CD markers whose potentials are better tested at sorting could be further identified for providing more clinically relevant cell products.

PERSPECTIVE

To enlarge the application of ASC-subset cellular products in clinics, storage of the efficient numbers of the individual cell population is the preliminary step. Future research related to the effects of the freezing-thawing process on the ASC subset, including safety, efficacy, potency, *etc*., would be of great value to better prepare the related cellular products.

The additional transcriptional analysis is crucial to understand the behaviors underlying the individual immunophenotypes from gene level; additional mechanism analysis is advantageous to fully validate the prominent ASC populations in our studies, such as immunoregulatory effects. Such studies will provide more useful evidence to fully comprehend the relationship between biological potency and immunophenotypes, allowing us to better target these ASC populations with the greatest therapeutic value.

We focus on the conventional 2-dimensional expansion that is currently used for clinical studies. However, growing evidence has highlighted the beneficial roles of the three-dimensional hydrogel model or alternative culturing substrates in tissue and organ transplantation over the last few decades. For example, tissue-engineered ASCs can retain stemness while exhibiting increased anti-inflammatory/immunomodulatory properties, robust survival/anti-apoptotic ability[151], or rapid cell growth rate[152]. Regrading ASC-subset transplantation (our primary focus), Ferraro et al.[80] have reported that human CD34/CD90 ASCs and collage scaffold constructs build loos connective and adipose tissue fabricated after *in vivo* transplantation. This will pave the way for further research into the novel delivery method for ASC-subsets-based grafts, which could cascade amplify their beneficial effects in regenerative medicine.

LITERATURE LIST

1. Afflerbach AK, Kiri MD, Detinis T, Maoz BM. Mesenchymal stem cells as a promising cell source for integration in novel in vitro models. Biomolecules. 2020;10:1–30.

2. Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW. Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects. Cryobiology. 2015;71:181–97.

3. Ullah I, Subbarao RB, Rho GJ. Human mesenchymal stem cells - Current trends and future prospective. Biosci Rep. 2015;35:191.

4. Krampera M, Franchini M, Pizzolo G, Aprili G. Mesenchymal stem cells: From biology to clinical use. Blood Transfus. 2007;5:120–9.

5. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211–28.

6. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13:4279–95.

7. Sheykhhasan M, Qomi RT, Ghiasi M. Fibrin Scaffolds Designing in order to Human Adipose-derived Mesenchymal Stem Cells Differentiation to Chondrocytes in the Presence of TGF-β3. Int J Stem Cells. 2015;

8. Bajek A, Gurtowska N, Olkowska J, Kazmierski L, Maj M, Drewa T. Adipose-Derived Stem Cells as a Tool in Cell-Based Therapies. Arch Immunol Ther Exp (Warsz). 2016;64:443–54.

9. Lee JM, Jung J, Lee HJ, et al. Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. Int Immunopharmacol. 2012;13:219–24.

10. Zhao Q, Ren H, Han Z. Mesenchymal stem cells: Immunomodulatory capability and clinical potential in immune diseases. J Cell Immunother. 2016;

11. Chen S, Cui G, Peng C, et al. Transplantation of adipose-derived mesenchymal stem cells attenuates pulmonary fibrosis of silicosis via anti-inflammatory and anti-apoptosis effects in rats. Stem Cell Res Ther. 2018;9:110–22.

12. Sheykhhasan M, Qomi RT, Kalhor N, Mehdizadeh M, Ghiasi M. Evaluation of the ability of natural and synthetic scaffolds in providing an appropriate

environment for growth and chondrogenic differentiation of adipose-derived mesenchymal stem cel. Indian J Orthop. 2015;

13. Álvarez-Viejo M. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. World J Stem Cells. 2015;

14. Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Vol. 21, Stem Cells and Development. 2012. p. 2724–52.

15. Fathi E, Farahzadi R. Isolation, culturing, characterization and aging of adipose tissue-derived mesenchymal stem cells: A brief overview. Brazilian Arch Biol Technol. 2016;

16. Kuo Y-R, Wang C-T, Cheng J-T, Kao G-S, Chiang Y-C, Wang C-J. Adipose-Derived Stem Cells Accelerate Diabetic Wound Healing Through the Induction of Autocrine and Paracrine Effects. Cell Transplant. 2016;25:71–81.

17. Takehara Y, Yabuuchi A, Ezoe K, et al. The restorative effects of adipose-derived mesenchymal stem cells on damaged ovarian function. Lab Investig. 2013;93:181–93.

18. Stepien A, Dabrowska NL, Maciagowska M, et al. Clinical Application of Autologous Adipose Stem Cells in Patients with Multiple Sclerosis: Preliminary Results. Mediators Inflamm. 2016;2016.

19. Mundra V, Gerling IC, Mahato RI. Mesenchymal stem cell-based therapy. Mol Pharm. 2013;10:77–89.

20. Gomes ME, Rada T, Reis RL. Adipose tissue-derived stem cells and their application in bone and cartilage tissue engineering. Tissue Engineering - Part B: Reviews. 2009.

21. Patrick CW. Breast Tissue Engineering. Annu Rev Biomed Eng. 2004;6:109–30.

22. Schäffler A, Büchler C. Concise Review: Adipose Tissue-Derived Stromal Cells-Basic and Clinical Implications for Novel Cell-Based Therapies. Stem Cells. 2007;25:818–27.

23. Lin G, Garcia M, Ning H, et al. Defining stem and progenitor cells within adipose tissue. Stem Cells Dev. 2008;

24. Corselli M, Chen CW, Crisan M, Lazzari L, Péault B. Perivascular ancestors of adult multipotent stem cells. Arteriosclerosis, Thrombosis, and Vascular Biology. 2010.

25. Lin CS, Xin ZC, Deng CH, Ning H, Lin G, Lue TF. Defining adipose tissue-derived stem cells in tissue and in culture. Histology and Histopathology. 2010.

26. Zimmerlin L, Donnenberg VS, Rubin JP, Donnenberg AD. Mesenchymal markers on human adipose stem/progenitor cells. Cytom Part A. 2013;83 A:134–40.

27. Zimmerlin L, Donnenberg VS, Pfeifer ME, et al. Stromal vascular progenitors in adult human adipose tissue. Cytom Part A. 2010;

28. Traktuev DO, Merfeld-Clauss S, Li J, et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res. 2008;102:77–85.

29. Crisan M, Chen CW, Corselli M, Andriolo G, Lazzari L, Péault B. Perivascular multipotent progenitor cells in human organs. In: Annals of the New York Academy of Sciences. 2009. p. 118–23.

30. Crisan M, Yap S, Casteilla L, et al. A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell. 2008;3:301–13.

31. Peinado JR, Pardo M, De la Rosa O, Malagón MM. Proteomic characterization of adipose tissue constituents, a necessary step for understanding adipose tissue complexity. Vol. 12, Proteomics. 2012. p. 607–20.

32. Tsuji W, Rubin JP, Marra KG. Adipose-derived stem cells: Implications in tissue regeneration. World J Stem Cells. 2014;6:312–21.

33. Nunes SS, Maijub JG, Krishnan L, et al. Generation of a functional liver tissue mimic using adipose stromal vascular fraction cell-derived vasculatures. Sci Rep. 3:2141–8.

34. Riordan NH, Ichim TE, Min WP, et al. Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. J Transl Med. 2009;7.

35. Koh YJ, Koh BI, Kim H, et al. Stromal vascular fraction from adipose tissue forms profound vascular network through the dynamic reassembly of blood

endothelial cells. Arterioscler Thromb Vasc Biol. 2011;

36. Dong Z, Peng Z, Chang Q, Lu F. The survival condition and immunoregulatory function of adipose stromal vascular fraction (SVF) in the early stage of nonvascularized adipose transplantation. PLoS One. 2013;

37. Klar AS, Güven S, Zimoch • Jakub, et al. Characterization of vasculogenic potential of human adipose-derived endothelial cells in a three-dimensional vascularized skin substitute.

38. Silva KR, Liechocki S, Carneiro JR, et al. Stromal-vascular fraction content and adipose stem cell behavior are altered in morbid obese and post bariatric surgery ex-obese women. Stem Cell Res Ther. 2015;

39. Yoshimura K, Shigeura T, Matsumoto D, et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol. 2006;208:64–76.

40. Bora P, Majumdar AS. Adipose tissue-derived stromal vascular fraction in regenerative medicine: a brief review on biology and translation.

41. Miranville A, Heeschen C, Sengenès C, Curat CA, Busse R, Bouloumié A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation. 2004;110:349–55.

42. Astori G, Vignati F, Bardelli S, et al. “In vitro” and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med. 2007;5:55.

43. Brzoska M, Geiger H, Gauer S, Baer P. Epithelial differentiation of human adipose tissue-derived adult stem cells. Biochem Biophys Res Commun. 2005;330:142–50.

44. Bourin P, Bunnell BA, Casteilla L, et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International So. Cytotherapy. 2013;15:641–8.

45. Andia I, Maffulli N, Burgos-Alonso N. Stromal vascular fraction technologies and clinical applications. Expert Opin Biol Ther. 2019;19:1289–305.

46. Cohen SR, Hewett S, Ross L, et al. Regenerative Cells For Facial Surgery:

Biofilling and Biocontouring. Aesthetic Surg J. 2017;37:S16–32.

47. Bianchi F, Maioli M, Leonardi E, et al. A New Nonenzymatic Method and Device to Obtain a Fat Tissue Derivative Highly Enriched in Pericyte-Like Elements by Mild Mechanical Forces From Human Lipoaspirates. Cell Transplant. 2013;22:2063–77.

48. Corselli M, Crisan M, Murray IR, et al. Identification of perivascular mesenchymal stromal/stem cells by flow cytometry. Cytom Part A. 2013;83A:714–20.

49. Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M, et al. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy. 2006;

50. Van Harmelen V, Skurk T, Röhrig K, et al. Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. Int J Obes. 2003;

51. Van Harmelen V, Röhrig K, Hauner H. Comparison of Proliferation and Differentiation Capacity of Human Adipocyte Precursor Cells from the Omental and Subcutaneous Adipose Tissue Depot of Obese Subjects. Metabolism. 2004;

52. Bakker AHF, Van Dielen FMH, Greve JWM, Adam JA, Buurman WA. Preadipocyte number in omental and subcutaneous adipose tissue of obese individuals. Obes Res. 2004;

53. Jurgens WJFM, Oedayrajsingh-Varma MJ, Helder MN, et al. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: Implications for cell-based therapies. Cell Tissue Res. 2008;332:415–26.

54. Riis S, Zachar V, Boucher S, Vemuri MC, Pennisi CP, Fink T. Critical steps in the isolation and expansion of adipose-derived stem cells for translational therapy. Expert Rev Mol Med. 2015;17:e11.

55. Gronthos S, Franklin DMMDM, Leddy HAH a a, Robey PGPGPG, Storms RWRWRW, Gimble JMJMM. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001;189:54–63.

56. Mitchell JB, McIntosh K, Zvonic S, et al. Immunophenotype of Human Adipose-Derived Cells: Temporal Changes in Stromal-Associated and Stem Cell-Associated Markers. Stem Cells. 2006;24:376–85.

57. Wan Safwani WKZ, Makpol S, Sathapan S, Chua KH. The changes of

stemness biomarkers expression in human adipose-derived stem cells during long-term manipulation. Biotechnol Appl Biochem. 2011;

58. Baer PC, Kuçi S, Krause M, et al. Comprehensive phenotypic characterization of human adipose-derived stromal/stem cells and their subsets by a high throughput technology. Stem Cells Dev. 2013;22:330–9.

59. Li CY, Wu XY, Tong JB, et al. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. Stem Cell Res Ther. 2015;

60. Dhanasekaran M, Indumathi S, Poojitha R, Kanmani A, Rajkumar JS, Sudarsanam D. Plasticity and banking potential of cultured adipose tissue derived mesenchymal stem cells. Cell Tissue Bank. 2013;

61. Mihaila SM, Frias AM, Pirraco RP, et al. Human Adipose Tissue-Derived SSEA-4 Subpopulation Multi-Differentiation Potential Towards the Endothelial and Osteogenic Lineages. Tissue Eng Part A. 2013;19:235–46.

62. Zannettino ACWCWW, Paton S, Arthur A, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. J Cell Physiol. 2008;214:413–21.

63. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. Cell Surface and Transcriptional Characterization of Human Adipose-Derived Adherent Stromal (hADAS) Cells. Stem Cells. 2005;23:412–23.

64. Peng Q, Alipour H, Porsborg S, Fink T, Zachar V. Evolution of asc immunophenotypical subsets during expansion in vitro. Int J Mol Sci. 2020;21:1408.

65. Zuk P. Adipose-Derived Stem Cells in Tissue Regeneration: A Review. ISRN Stem Cells. 2013;2013:1–35.

66. Li Q, Qi LJ, Guo ZK, Li H, Zuo HB, Li NN. CD73+ adipose-derived mesenchymal stem cells possess higher potential to differentiate into cardiomyocytes in vitro. J Mol Histol. 2013;

67. Rada T, Reis RL, Gomes ME. Distinct Stem Cells Subpopulations Isolated from Human Adipose Tissue Exhibit Different Chondrogenic and Osteogenic Differentiation Potential. Stem Cell Rev Reports. 2011;7:64–76.

68. Chung MT, Liu C, Hyun JS, et al. CD90 (Thy-1)-Positive Selection Enhances Osteogenic Capacity of Human Adipose-Derived Stromal Cells. Tissue Eng

Part A. 2013;19:989–97.

69. Ishimura D, Yamamoto N, Tajima K, et al. Differentiation of adipose-derived stromal vascular fraction culture cells into chondrocytes using the method of cell sorting with a mesenchymal stem cell marker. Tohoku J Exp Med. 2008;

70. Jiang T, Liu W, Lv X, et al. Potent in vitro chondrogenesis of CD105 enriched human adipose-derived stem cells. Biomaterials. 2010;31:3564–71.

71. Yamamoto N, Akamatsu H, Hasegawa S, et al. Isolation of multipotent stem cells from mouse adipose tissue. J Dermatol Sci. 2007;

72. Quirici N, Scavullo C, De Girolamo L, et al. Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. Stem Cells Dev. 2010;19:915–25.

73. Suga H, Matsumoto D, Eto H, et al. Functional implications of CD34 expression in human adipose-derived stem/progenitor cells. Stem Cells Dev. 2009;18:1201–9.

74. Doornaert MAJ, Declercq H, Stillaert F, et al. Intrinsic dynamics of the fat graft: In vitro interactions between the main cell actors. Plast Reconstr Surg. 2012;130:1001–9.

75. De Francesco F, Tirino V, Desiderio V, et al. Human CD34 + /CD90 + ASCs Are Capable of Growing as Sphere Clusters, Producing High Levels of VEGF and Forming Capillaries. PLoS One. 2009;4:e6537.

76. Levi B, Wan DC, Glotzbach JP, et al. CD105 protein depletion enhances human adipose-derived stromal cell osteogenesis through reduction of transforming growth factor β1 (TGF-β1) signaling. J Biol Chem. 2011;286:39497–509.

77. Banas A, Teratani T, Yamamoto Y, et al. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. Hepatology. 2007;

78. Rada T, Carvalho P, Santos T, Castro A, Reis R, Gomes M. Chondrogenic Potential of Two hASCs Subpopulations Loaded onto Gellan Gum Hydrogel Evaluated in a Nude Mice Model. Curr Stem Cell Res Ther. 2013;

79. Xiao J, Yang X, Jing W, et al. Adipogenic and osteogenic differentiation of Lin-CD271 +Sca-1+ adipose-derived stem cells. Mol Cell Biochem. 2013;377:107–19.

80. Ferraro GA, De Francesco F, Nicoletti G, et al. Human adipose CD34+CD90+ stem cells and collagen scaffold constructs grafted in vivo fabricate loose connective and adipose tissues. J Cell Biochem. 2013;114:1039–49.

81. Borrelli MR, Patel RA, Blackshear C, et al. CD34+CD146+ adipose-derived stromal cells enhance engraftment of transplanted fat. Stem Cells Transl Med. 2020;9:1389–400.

82. Borrelli MR, Patel RA, Adem S, et al. The antifibrotic adipose-derived stromal cell: Grafted fat enriched with CD74+ adipose-derived stromal cells reduces chronic radiation-induced skin fibrosis. Stem Cells Transl Med. 2020;9:1401–13.

83. Riis S, Nielsen FM, Pennisi CP, Zachar V, Fink T. Comparative Analysis of Media and Supplements on Initiation and Expansion of Adipose-Derived Stem Cells. Stem Cells Transl Med. 2016;5:314–24.

84. Wang F, Zachar V, Pennisi CP, Fink T, Maeda Y, Emmersen J. Hypoxia enhances differentiation of adipose tissue-derived stem cells toward the smooth muscle phenotype. Int J Mol Sci. 2018;19:517.

85. Munir S, Foldager CB, Lind M, Zachar V, Søballe K, Koch TG. Hypoxia enhances chondrogenic differentiation of human adipose tissue-derived stromal cells in scaffold-free and scaffold systems. Cell Tissue Res. 2014;355:89–102.

86. Fink SR ;Rhonda NHIJIADKSBMCVCPPVZ, Riis S, Newman R, et al. Hypoxia enhances the wound-healing potential of adipose-derived stem cells in a novel human primary keratinocyte-based scratch assay. Int J Mol Med. 2017;39:587–94.

87. Yang S, Pilgaard L, Chase LG, et al. Defined xenogeneic-free and hypoxic environment provides superior conditions for long-term expansion of human adipose-derived stem cells. Tissue Eng - Part C Methods. 2012;18:593–602.

88. Minonzio G, Corazza M, Mariotta L, et al. Frozen adipose-derived mesenchymal stem cells maintain high capability to grow and differentiate q. Cryobiology. 2014;69:211–6.

89. Devitt SM, Carter CM, Dierov R, Weiss S, Gersch RP, Percec I. Successful isolation of viable adipose-derived stem cells from human adipose tissue subject to long-term cryopreservation: Positive implications for adult stem cell-based therapeutics in patients of advanced age. Stem Cells Int. 2015;

90. CytoFLEX, Research Flow Cytometry [Internet]. [Accessed 2021 Mar 25]. Available from: https://www.beckman.com/flow-cytometry/instruments/cytoflex

91. Bishop JE, Dickerson J, Stall A, et al. A Setup System for Compensation: BD CompBeads plus BD FACSDiva Software.

92. Beavis AJ, Pennline KJ. ALLO-7: A new fluorescent tandem dye for use in flow cytometry. Cytometry. 1996;24:390–4.

93. Forman MA, Gupta RK. Tandem dyes for flow cytometry: can we overcome quality concerns? MLO Med Lab Obs. 2007;

94. Roederer M. Spectral compensation for flow cytometry: Visualization artifacts, limitations, and caveats. Cytometry. 2001;45:194–205.

95. Szalóki G, Goda K. Compensation in multicolor flow cytometry. Cytom Part A. 2015;87:982–5.

96. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nat Protoc 2012 712. 2012;7:2067–79.

97. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality assurance for polychromatic flow cytometry. Nat Protoc. 2006;

98. Nielsen FM, Riis SE, Andersen JI, et al. Discrete adipose-derived stem cell subpopulations may display differential functionality after in vitro expansion despite convergence to a common phenotype distribution. Stem Cell Res Ther. 2016;7:177.

99. Sample Preparation for Sorting | Flow Cytometry [Internet]. [Accessed 2021 Sep 29]. Available from: https://medicine.uiowa.edu/flowcytometry/protocolssample-prep/sample-preparation-sorting

100. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–7.

101. 4 Methods for Measuring Cell Proliferation [Internet]. [Accessed 2021 Aug 19]. Available from: https://blog.abclonal.com/blog/4-methods-for-measuring-cell-proliferation

102. Fujiwara O, Prasai A, Perez-Bello D, et al. Adipose-derived stem cells improve grafted burn wound healing by promoting wound bed blood flow. Burn Trauma. 2020;8:9.

103. Li P, Guo X. A review: therapeutic potential of adipose-derived stem cells in cutaneous wound healing and regeneration. Stem Cell Res Ther. 2018;9.

104. LV T, K R-S, AL M, MJ T. Adipose Tissue: A Source of Stem Cells with Potential for Regenerative Therapies for Wound Healing. J Clin Med. 2020;9:2161.

105. Aalborg Universitet Adipose-derived stem cells for treatment of chronic wounds.

106. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9:239–52.

107. Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. J Invest Dermatol. 2017;137:e11–6.

108. Augello A, De Bari C. Mesenchymal stem cells: A perspective from in vitro cultures to in vivo migration and niches. Eur Cells Mater. 2010;20:121–33.

109. Shu W, Shu YT, Dai CY, Zhen QZ. Comparing the biological characteristics of adipose tissue-derived stem cells of different persons. J Cell Biochem. 2012;113:2020–6.

110. Wu W, Niklason L, Steinbacher DM. The Effect of Age on Human Adipose-Derived Stem Cells. Plast Reconstr Surg. 2013;131:27–37.

111. Liu M, Lei H, Dong P, et al. Adipose-Derived Mesenchymal Stem Cells from the Elderly Exhibit Decreased Migration and Differentiation Abilities with Senescent Properties. Cell Transplant. 2017;26:1505–19.

112. Frazier TP, Gimble JM, Devay JW, Tucker HA, Chiu ES, Rowan BG. Body mass index affects proliferation and osteogenic differentiation of human subcutaneous adipose tissue-derived stem cells. BMC Cell Biol. 2013;14.

113. Faustini M, Bucco M, Chlapanidas T, et al. Nonexpanded mesenchymal stem cells for regenerative medicine: Yield in stromal vascular fraction from adipose tissues. Tissue Eng - Part C Methods. 2010;

114. Mojallal A, Lequeux C, Shipkov C, et al. Influence of age and body mass

index on the yield and proliferation capacity of Adipose-derived stem cells. Aesthetic Plast Surg. 2011;

115. Ferrer-Lorente R, Bejar MT, Tous M, Vilahur G, Badimon L. Systems biology approach to identify alterations in the stem cell reservoir of subcutaneous adipose tissue in a rat model of diabetes: Effects on differentiation potential and function. Diabetologia. 2014;

116. Cianfarani F, Toietta G, Di Rocco G, Cesareo E, Zambruno G, Odorisio T. Diabetes impairs adipose tissue-derived stem cell function and efficiency in promoting wound healing. Wound Repair Regen. 2013;

117. Rodriguez-Menocal L, Salgado M, Ford D, Van Badiavas E. Stimulation of Skin and Wound Fibroblast Migration by Mesenchymal Stem Cells Derived from Normal Donors and Chronic Wound Patients. Stem Cells Transl Med. 2012;

118. Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP. Regional anatomic and age effects on cell function of human adipose-derived stem cells. In: Annals of Plastic Surgery. 2008.

119. Di Taranto G, Cicione C, Visconti G, et al. Qualitative and quantitative differences of adipose-derived stromal cells from superficial and deep subcutaneous lipoaspirates: A matter of fat. Cytotherapy. 2015;

120. Domenis R, Lazzaro L, Calabrese S, et al. Adipose tissue derived stem cells: In vitro and in vivo analysis of a standard and three commercially available cell-assisted lipotransfer techniques. Stem Cell Res Ther. 2015;

121. Zielins ER, Brett EA, Longaker MT, Wan DC. Autologous Fat Grafting: The Science behind the Surgery. Aesthetic Surgery Journal. 2016.

122. Palumbo P, Lombardi F, Siragusa G, Grazia Cifone M, Cinque B, Giuliani M. Molecular Sciences Methods of Isolation, Characterization and Expansion of Human Adipose-Derived Stem Cells (ASCs): An Overview. Int J Mol. 2018;19:1897.

123. Busser H, De Bruyn C, Urbain F, et al. Isolation of adipose-derived stromal cells without enzymatic treatment: Expansion, phenotypical, and functional characterization. Stem Cells Dev. 2014;

124. Palumbo P, Miconi G, Cinque B, et al. In vitro evaluation of different methods of handling human liposuction aspirate and their effect on adipocytes and adipose derived stem cells. J Cell Physiol. 2015;

125. Fossett E, Khan WS. Optimising human mesenchymal stem cell numbers for clinical application: A literature review. Stem Cells International. 2012.

126. Neri S, Bourin P, Peyrafitte JA, Cattini L, Facchini A, Mariani E. Human Adipose Stromal Cells (ASC) for the Regeneration of Injured Cartilage Display Genetic Stability after In Vitro Culture Expansion. PLoS One. 2013;

127. Gadelkarim M, Abushouk AI, Ghanem E, Hamaad AM, Saad AM, Abdel-Daim MM. Adipose-derived stem cells: Effectiveness and advances in delivery in diabetic wound healing. Vol. 107, Biomedicine and Pharmacotherapy. Elsevier Masson SAS; 2018. p. 625–33.

128. Hassanshahi A, Hassanshahi M, Khabbazi S, et al. Adipose-derived stem cells for wound healing. J Cell Physiol. 2019;234:7903–14.

129. Baer PC, Griesche N, Luttmann W, Schubert R, Luttmann A, Geiger H. Human adipose-derived mesenchymal stem cells in vitro: evaluation of an optimal expansion medium preserving stemness. Taylor Fr. 2009;12:96–106.

130. Cramer C, Freisinger E, Jones RK, et al. Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. Stem Cells Dev. 2010;19:1875–84.

131. López M, Bollag RJ, Yu JC, Isales CM, Eroglu A. Chemically defined and xeno-free cryopreservation of human adipose-derived stem cells. PLoS One. 2016;11.

132. Halme DG, Kessler DA. FDA regulation of stem-cell-based therapies. N Engl J Med. 2006;

133. James AW, Levi B, Nelson ER, et al. Deleterious effects of freezing on osteogenic differentiation of human adipose-derived stromal cells in vitro and in vivo. Stem Cells Dev. 2011;20:427–39.

134. Yong KW, Pingguan-Murphy B, Xu F, et al. Phenotypic and Functional Characterization of Long-Term Cryopreserved Human Adipose-derived Stem Cells. Sci Rep. 2015;5:1–10.

135. Shaik S, Wu X, Gimble J, Devireddy R. Effects of Decade Long Freezing Storage on Adipose Derived Stem Cells Functionality. Sci Rep. 2018;8:1–12.

136. Agostini F, Rossi FM, Aldinucci D, et al. Improved GMP compliant approach to manipulate lipoaspirates, to cryopreserve stromal vascular fraction, and to expand adipose stem cells in xeno-free media. Stem Cell Res Ther. 2018;9:1–

16.

137. Dominici M, Paolucci P, Conte P, Horwitz EM. Heterogeneity of Multipotent Mesenchymal Stromal Cells: From Stromal Cells to Stem Cells and Vice Versa. Transplantation. 2009;87:S36–42.

138. Smith RJ, Reid AJ. The potential of adipose-derived stem cell subpopulations in regenerative medicine. Regen Med. 2018;13:357–60.

139. Baer PC, Geiger H. Adipose-Derived Mesenchymal Stromal/Stem Cells: Tissue Localization, Characterization, and Heterogeneity. Stem Cells Int. 2012;2012:11.

140. Wu CC, Liu FL, Sytwu HK, Tsai CY, Chang DM. CD146+ mesenchymal stem cells display greater therapeutic potential than CD146- cells for treating collagen-induced arthritis in mice. Stem Cell Res Ther. 2016;7.

141. Espagnolle N, Guilloton F, Deschaseaux F, et al. CD146 expression on mesenchymal stem cells is associated with their vascular smooth muscle commitment. J Cell Mol Med. 2014;18:104–14.

142. Lauvrud AT, Kelk P, Wiberg M, Kingham PJ. Characterization of human adipose tissue-derived stem cells with enhanced angiogenic and adipogenic properties. J Tissue Eng Regen Med. 2017;11:2490–502.

143. Borrelli MR, Adem S, Deleon Diaz NM, et al. The Proangiogenic Adipose-derived Stromal Cell (ASC): Cd146+ Ascsenhance The Regenerative Effects Of Grafted Fat. Plast Reconstr Surg. 2020;8:28–9.

144. Sacchetti B, Funari A, Michienzi S, et al. Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. Cell. 2007;131:324–36.

145. SM W, F G, M van der G, et al. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. Br Med Bull. 2013;108:25–53.

146. Armulik A, Abramsson A, Betsholtz C. Endothelial/Pericyte Interactions. Circ Res. 2005;97:512–23.

147. A C-G, I W, I G, et al. Comparison of 3 techniques of fat grafting and cell-supplemented lipotransfer in athymic rats: a pilot study. Aesthetic Surg J. 2013;33:713–21.

148. K M, KJ F, AA W, YS C, WA M, RJ D. Adipose-derived stem cells promote angiogenesis and tissue formation for in vivo tissue engineering. Tissue Eng Part A. 2013;19:1327–35.

149. Calabrese G, Giuffrida RR, Lo Furno D, et al. Potential effect of CD271 on human mesenchymal stromal cell proliferation and differentiation. Int J Mol Sci. 2015;16:15609–24.

150. Brett E, Zielins ER, Chin M, et al. Isolation of CD248-expressing stromal vascular fraction for targeted improvement of wound healing. Wound Repair Regen. 2017;25:414–22.

151. Petrenko Y, Syková E, Kubinová Š. The therapeutic potential of three-dimensional multipotent mesenchymal stromal cell spheroids. Vol. 8, Stem Cell Research and Therapy. BioMed Central Ltd.; 2017. p. 94.

152. Dessels C, Durandt C, Pepper MS. Comparison of human platelet lysate alternatives using expired and freshly isolated platelet concentrates for adipose-derived stromal cell expansion. Platelets. 2019;

APPENDICES

Appendix A. Spectra view

All these figures below were created by FluoroFinder 2.0 online web tool.

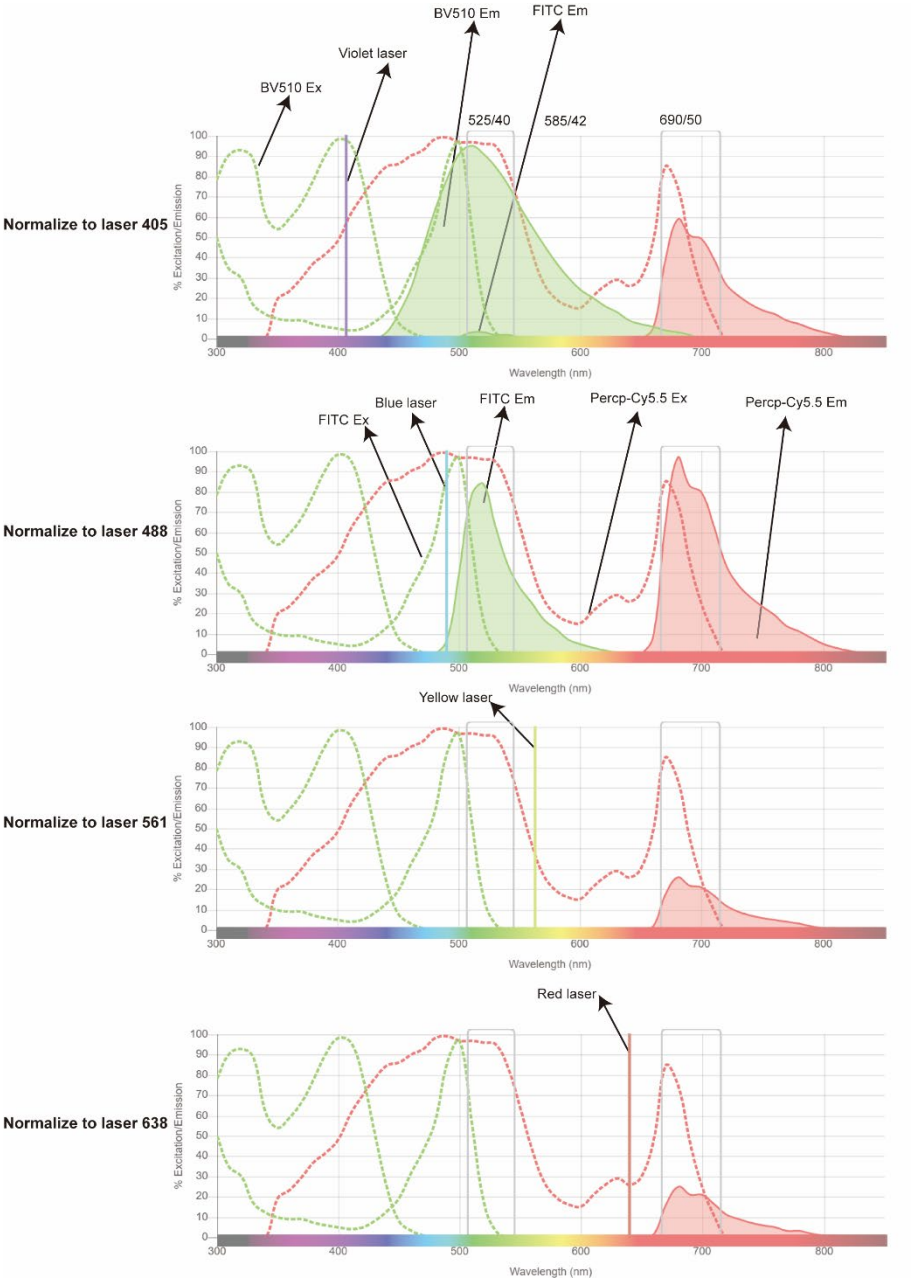


Figure A1: Spectra view of fluorescence used in Panel 1 of Study I. All the exciatThe annotations are only made when the dyes are detected in the targeted laser view, although some dyes could be excited by multiple lasers. Abbreviations: BP, bandpass; Ex, excitation; Em, emission.

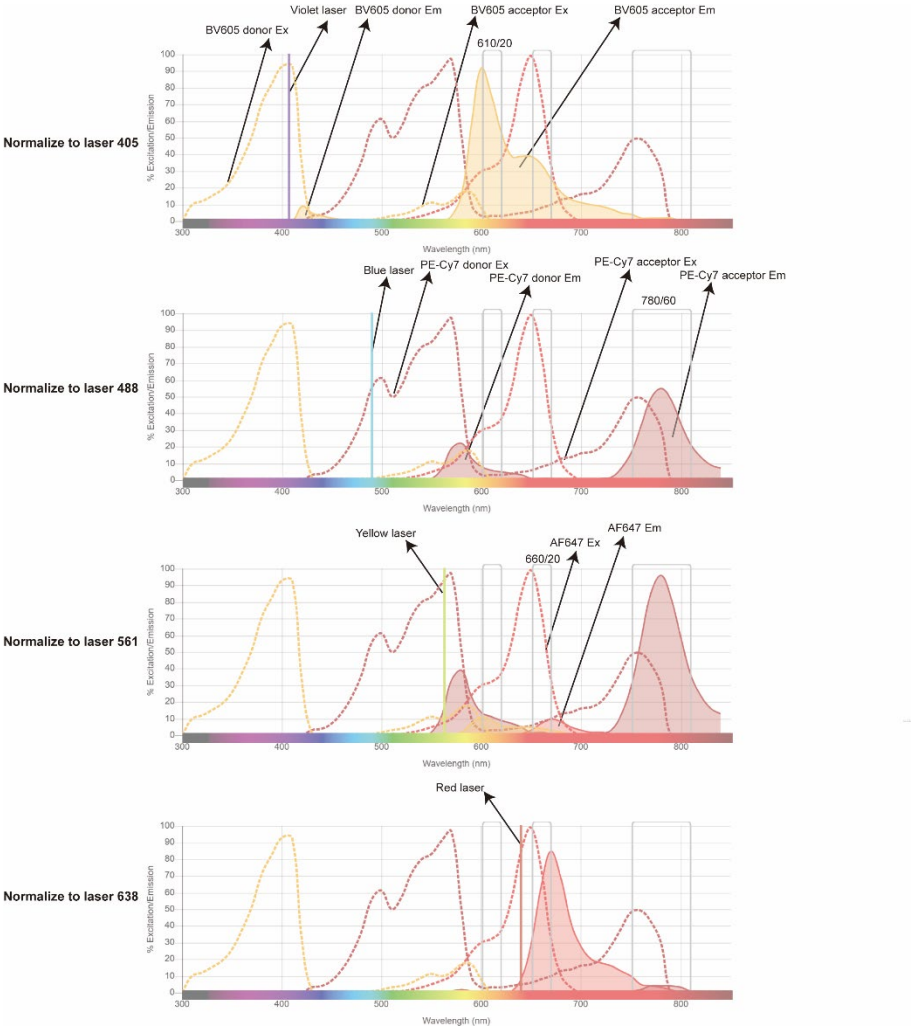


Figure A2: Spectra view of fluorescence used in Panel 2 of Study I.

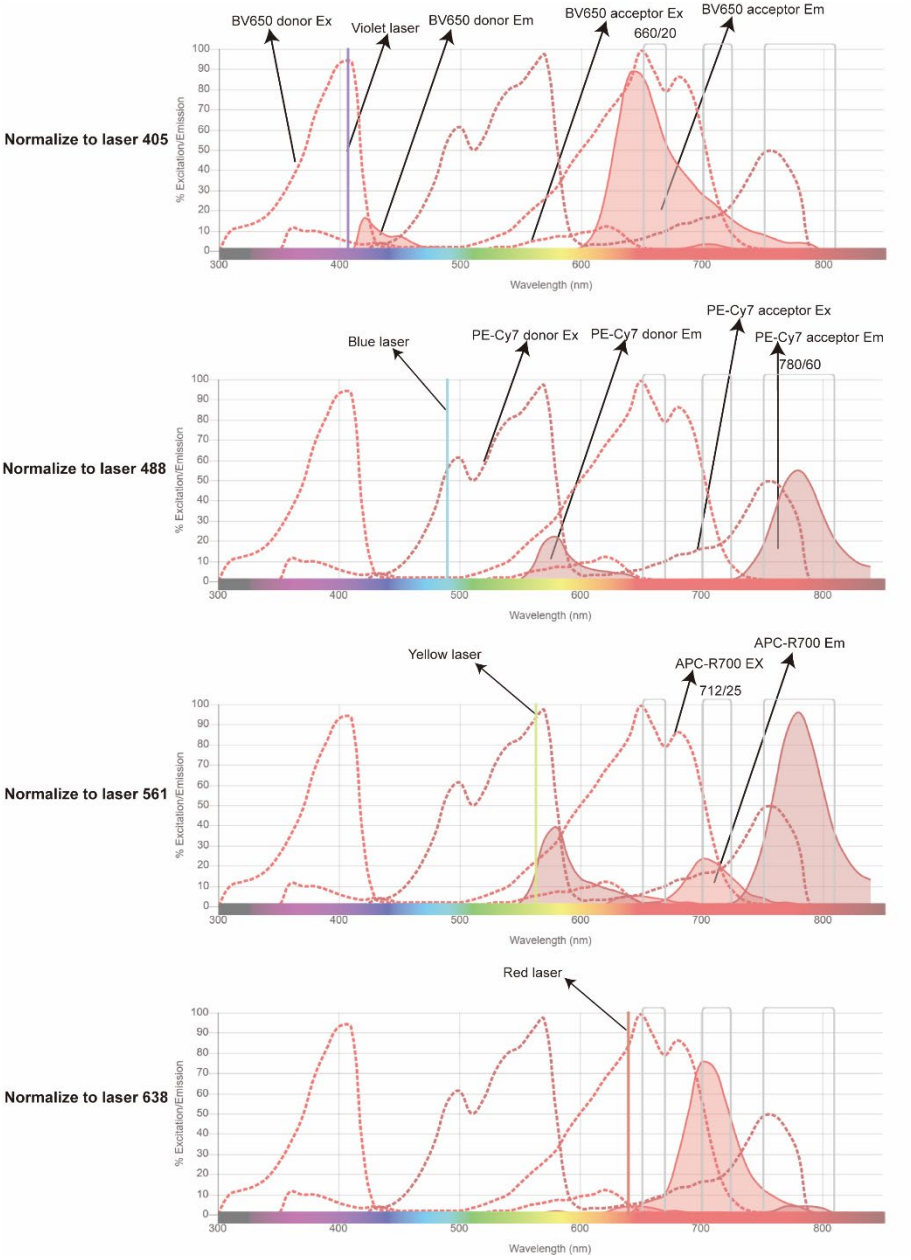


Figure A3: Spectra view of fluorescence used in Panel 3 of Study I.

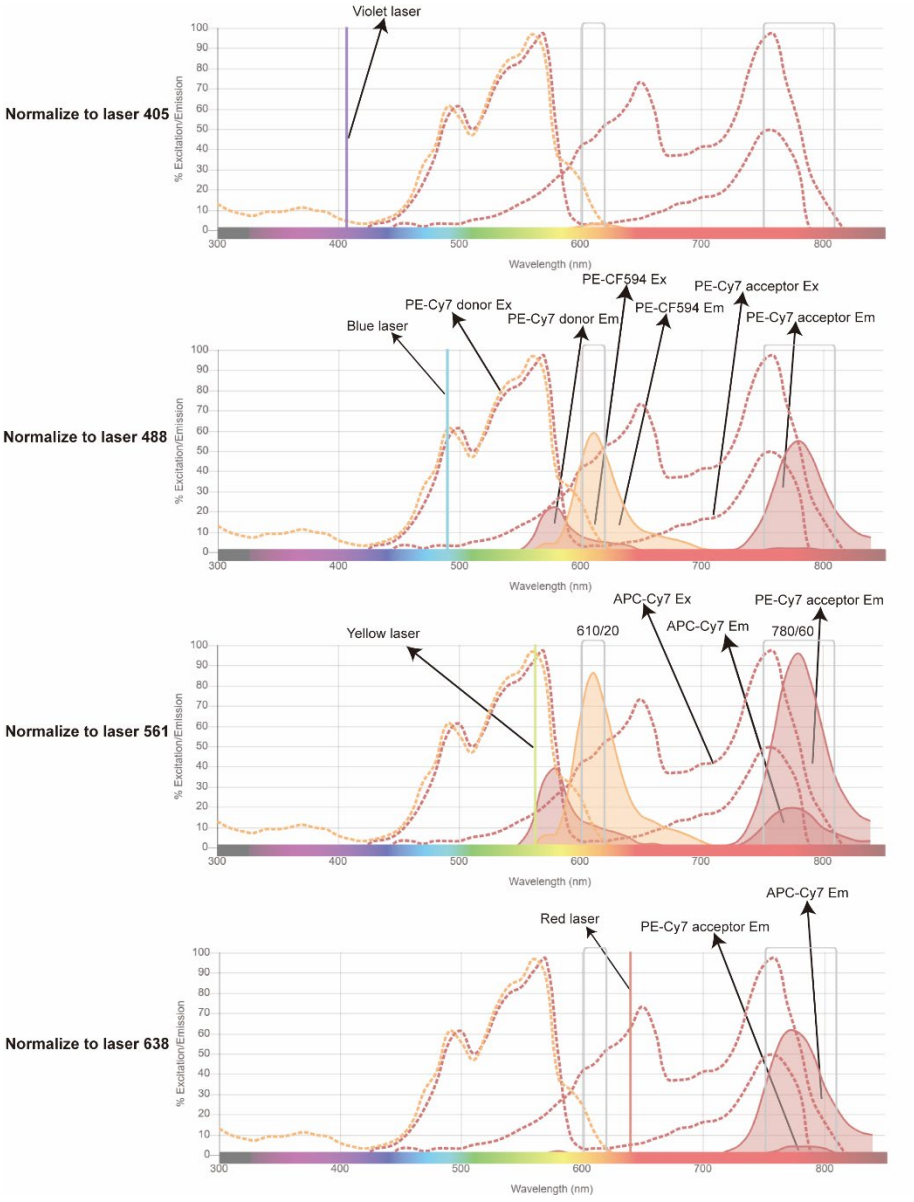


Figure A4: Spectra view of fluorescence used in Panel 4 of Study I.

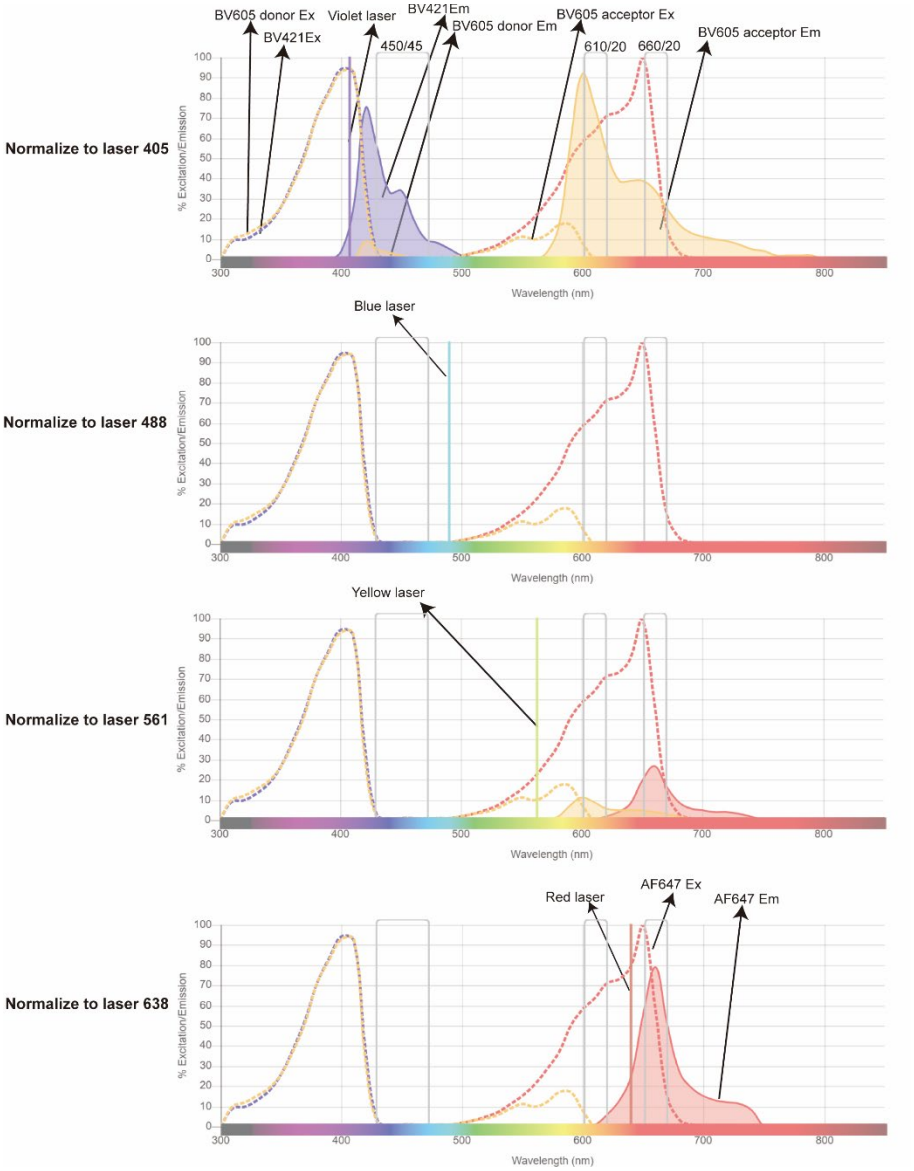


Figure A5: Spectra view of fluorescence used in Panel 5 of Study I.

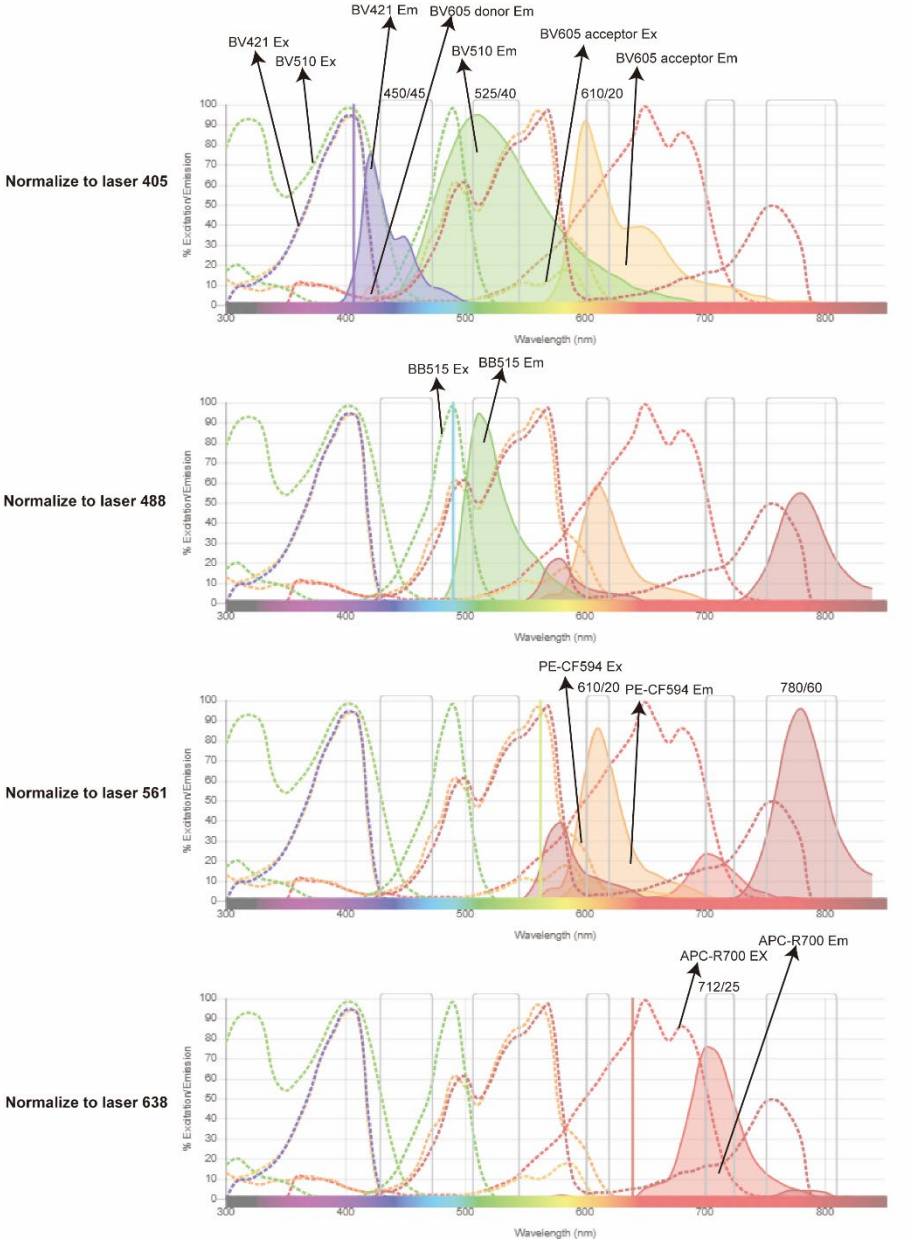


Figure A5: Spectra view of fluorescence used in Panel 1 of Study II.

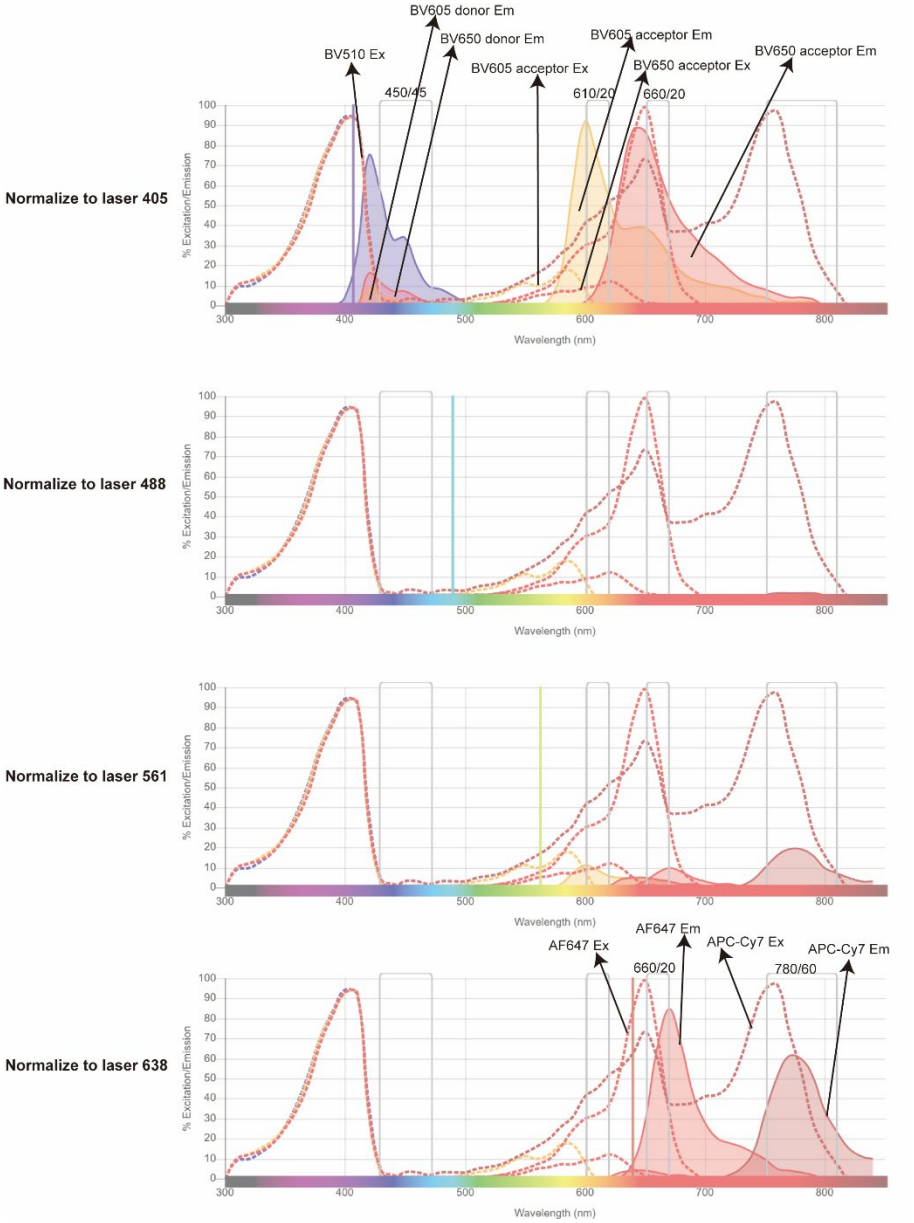


Figure A5: Spectra view of fluorescence used in Panel 2 of Study II

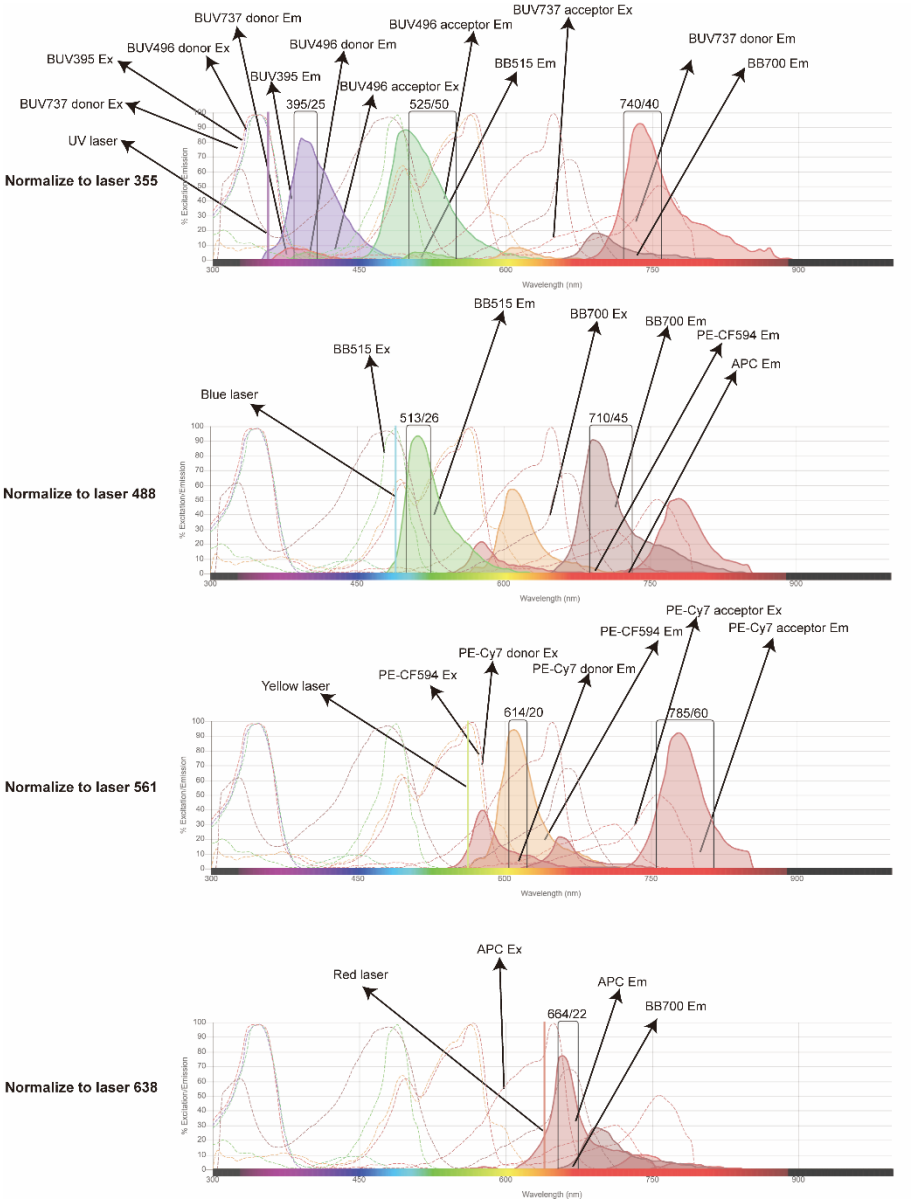


Figure A6: Spectra view of fluorescence used in Panel 1 of Study III

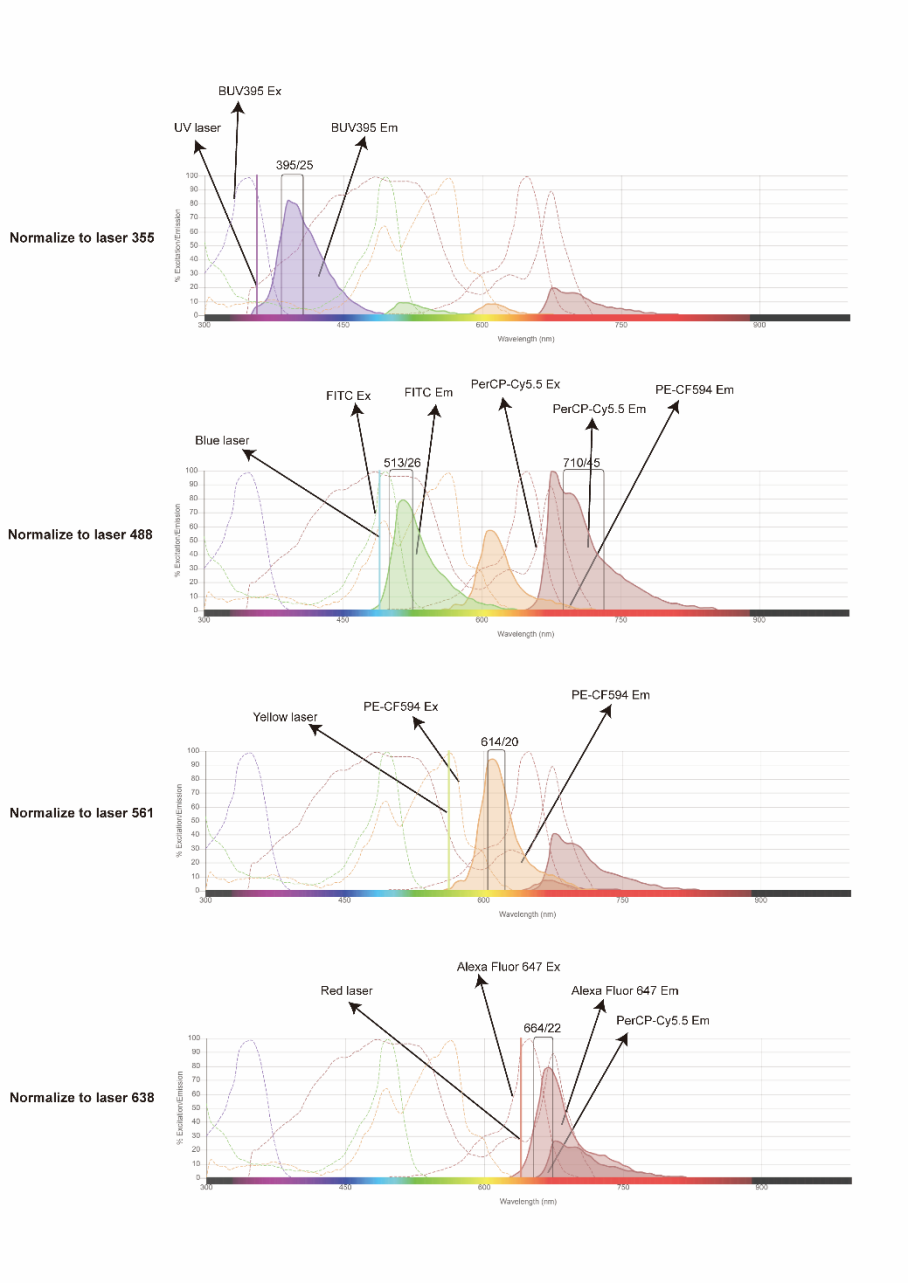


Figure A7: Spectra view of fluorescence used in Panel 2 of Study III

Appendix B. Manuscripts

ISSN (online): 2246-1302
ISBN (online): 978-87-7573-923-3

AALBORG UNIVERSITY PRESS