



HAL
open science

BIOPROCEDES DE CULTURE CELLULAIRE : APPLICATION A LA PRODUCTION DE VIRUS ET A LA MEDECINE REGENERATIVE

Emma Petiot

► **To cite this version:**

Emma Petiot. BIOPROCEDES DE CULTURE CELLULAIRE : APPLICATION A LA PRODUCTION DE VIRUS ET A LA MEDECINE REGENERATIVE. Biotechnology. Université Claude Bernard Lyon 1, 2020. <tel-04258278>

HAL Id: tel-04258278

<https://hal.science/tel-04258278v1>

Submitted on 25 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Année 2020

HABILITATION A DIRIGER DES RECHERCHES

présentée devant

l'Université Claude Bernard LYON I

BIOPROCEDES DE CULTURE CELLULAIRE :
APPLICATION A LA PRODUCTION DE VIRUS ET A LA MEDECINE
REGENERATIVE

SPECIALITE :
BIOCHIMIE & BIOPROCEDES

par
Dr. PETIOT Emma

Soutenance prévue le 22 octobre 2020 devant la Commission d'examen

ALVES Paula	Associate Professor	Examinatrice
COLL Jean-Luc	DR1 INSERM	Rapporteur
GODIA Francesc	Full Professor	Rapporteur
LINA Bruno	Professeur des Universités – Praticien Hospitalier	Rapporteur
OLMOS Eric	Professeur	Examineur
MALLEIN-GERIN Frederic	DR1 CNRS	Examineur

ICBMS – Gembas

Abbreviations

CVV : Candidate Vaccine Virus

ELISA : Enzyme-linked immunosorbent assay

fc : characteristic frequency

FDA : Food and Drug Administration

h.p.i. : hour post-infection

IEX Chromatography : Ion exchange chromatography

IVP : Infectious viral particles

Mabs : Monoclonal antibodies

MMP : Metalloproteinase enzyme

MRI : Magnetic Resonance Imaging

NIRS : Near Infrared Spectroscopy

PAT : Process Analytical Technology

PFU : Plaque forming unit

QbD: Quality by Design

SRID : Single Radia Immunodiffusion assay

TCID₅₀ : Median Tissue culture infectious dose (infectivity assay)

TRPS : Tunable Resistive Pulse Sensing

VCD : Viable cell density

Plan

Abbreviations.....	2
Résumé.....	4
Summary	5
1. Curriculum Vitae	7
2. Teaching and administrative activities.....	8
3. External activities.....	13
4. Past and present research activities.....	15
5. Project: Bioprinted tissue bioprocesses	46
6. Scientific production.....	54
7. Literature.....	Error! Bookmark not defined.

Résumé

Les bioprocédés basés sur la culture cellulaire sont utilisés à grande échelle depuis les années 80 pour les opérations de bio-fabrication. Désormais, ils sont utilisés pour un large éventail d'applications dont la production de molécules thérapeutiques (protéine recombinante, anticorps monoclonal) ou de vaccins (vaccin viral), les thérapies cellulaires (cellules souches, cellules CART avec vecteurs viraux), et plus récemment la production de tissus vivants pour la médecine régénérative. En général, les procédés à base de cellules sont hautement technologiques et nécessitent un large éventail d'expertises pour permettre une production contrôlée, et maîtriser la qualité des produits. Les domaines scientifiques et les expertises qui sous-tendent le développement de tels processus sont divers et impliquent une forte pluridisciplinarité.

De nos jours, les approches multi-échelles sont nécessaires pour relier la physiologie cellulaire au métabolisme, l'interaction du produit avec les comportements intracellulaires (ex. Cinétique de réplication virale), la différenciation cellulaire avec le processus et l'équipement utilisé / mis en œuvre pour fournir et maintenir des conditions optimales de culture cellulaire. En plus de ces considérations, des outils analytiques et des méthodologies permettant la caractérisation et la quantification à la fois du devenir cellulaire et du produit généré sont essentiels pour atteindre des bioprocédés de haute qualité et en comprendre les mécanismes biologiques fondamentaux. Cette approche est principalement décrite dans la FDA et l'institution des agences de régulation comme la technologie d'analyse de la qualité par la conception et des processus.

Le manuscrit proposé décrit la stratégie de recherche et les applications que j'ai développées au cours de ces 10 dernières années sur les procédés de production à base de cellules et appliqués à plusieurs domaines d'application; à savoir le domaine de la vaccinologie avec les bioprocédés de production de vaccins viraux, les thérapies cellulaire / oncothérapies à base de bioprocédés de production de vecteurs viraux et le domaine de la médecine régénérative avec des bioprocédés de production et de maturation de tissus.

L'objectif est de présenter et d'obtenir une Habilitation Française à mener des recherches (HDR) pour poursuivre de telles activités de recherche et de former un groupe d'édition autour de ces aspects au sein de mon équipe de recherche actuelle.

Summary

Cell-culture based bioprocesses are in-use at large scale since the 80's for bio-manufacturing operations. They now concern a wide range of applications including the production of therapeutic molecules (recombinant protein, monoclonal antibody) or vaccines (viral vaccine), the cell-based therapies (stem cells, CART cells with viral vectors), and more recently the production of living tissues for regenerative medicine. In general, cell-based processes are highly technological and necessitate a large range of expertise to allow for controlled production of qualified products. Science and expertise underlying the development of such processes are thus several and imply multi-disciplinarity.

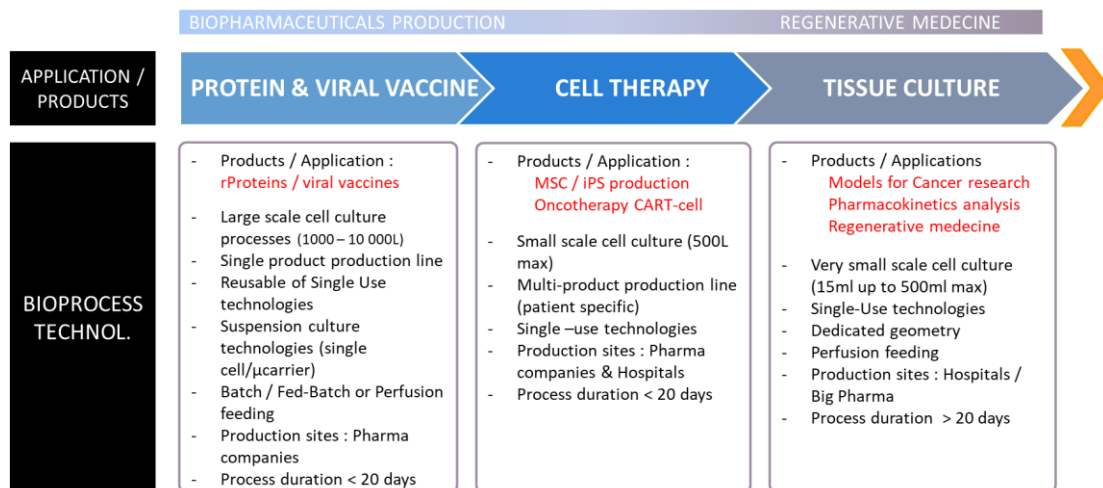


Figure 1_ Cell based bioprocesses: development history. *In red detailed targeted applications and product of each sectors.*

Nowadays, multi-scale approaches are necessary to link cell physiology with metabolism, product interaction with the intracellular behaviours (ex. viral replication kinetics), cell differentiation with the process and equipment operated/implemented to provide and maintain optimal cells culture conditions. On the top of such considerations, analytical tools and methodologies allowing the characterization and quantification of both cell fate and generated product are critical to reach high quality bioprocesses and a robust understanding of the fundamental biological mechanisms. This approach is mostly described by FDA and other regulation agencies institutions as the “Quality by Design” and “Process Analytical Technology” methodologies.

The proposed manuscript described the research strategy and applications I developed over the last 10 years on cell-based production processes applied to

several application fields; vaccinology field with viral vaccine bioprocesses, cell-therapy/oncotherapy with viral vectors production bioprocesses and regenerative medicine field with tissue production and maturation bioprocesses.

The objective is to present and obtain a French Habilitation to conduct research (HDR) to pursue such research activities and to form a publishing group around these aspects within my current research team.

1. Curriculum Vitae

Emma PETIOT, PhD

18th of January 1984 (37 years old)

– 20 publications, 14 oral presentations & 23 posters in international meetings –

H factor : 11

https://www.researchgate.net/profile/Emma_Petiot

EDUCATION

2006-2009	PHD in Cell culture Engineering / Bioprocessing - Institut National Polytechnique de Lorraine (INPL) – Nancy – France - Dr. Annie MARC & Dr. Hervé PINTON - Collaborative Project Sanofi Pasteur - LRGF
2005-2006	RESEARCH MASTER DEGREE in “Industrial Microbiology et Catalysis”- Institut National des Sciences Appliqués (INSA) - Toulouse
2001-2006	ENGINEER DEGREE in “ Biochemistry & Bioprocesses”- INSA Engineer school – Toulouse - France

CURRENT POSITION

<i>Scientist - Lecturer</i> Jan 2017 – CPE-Lyon : Laboratory ICBMS 3d.Fab -Lyon	Management of Bioprocess / Biotechnology teaching programs at CPE-Lyon Researcher at ICBMS / 3d. FAB platform- Development of bioreactor and in-line tools applied to bioprinted tissues.
--	--

PREVIOUS POSITIONS

<i>Researcher</i> Sept 2014 – Dec 2016 VirPath laboratory – University Claude Bernard Lyon (UCBL) -Lyon	Project coordinator of European Fellowship FP-7 : Marie-Curie Incoming Intranse Fellowship - Development of cell-based bioproduction platform for influenza virus and influenza-VLP : Production of Chimeric influenza-VLP used as vaccine platform for presentation of foreign antigens – Application to malaria - International consortium McGill – Canada / A.Star Singapour. Evaluation & optimization of influenza production process on novel avian cell line - <i>Collaboration with French Transgene company</i>
<i>Scientist - Lecturer</i> Nov 2013 – Aug. 2014 CPE-Lyon - VirPath laboratory (UCBL)-Lyon	Development of cell-based bioproduction platform and processes for influenza virus production. <i>Evaluation of new cell lines for influenza vaccine production.</i> Launch and management of a new Bioprocess teaching program at CPE-Lyon for post-graduate student and professionals at CPE-Lyon.
<i>Associate researcher</i> 2010 – 2013 Biotechnology Research Institute (IRB) - National Research Center (NRC) - Montréal	Team coordinator of influenza vaccine program - Development of an influenza vaccine production (<i>production – purification – quantification</i>) of wild-type virus or Virus Like Particles Management or Participation to industrial contracts - Consultancy for Oncolytics Biotech® company: <i>LC-MS/MS study for detection of Host-Cell protein of HEK293 cell in reovirus production process</i> Collaboration with MEDICAGO Inc. company: <i>Development of quantification techniques for influenza-VLPs made in plant cells.</i> Collaboration with PALL industry: <i>Co-development of purification strategies of influenza virus produced from suspension cell culture.</i>
PHD Student 2006 – 2009 CNRS - Sanofi-Pasteur Nancy / Lyon	Vero cell culture processes in serum-free medium: contribution to the development of PAT strategy. <i>Culture medium optimization – Metabolism study – Development of in-line monitoring tools of the process.</i>
CAREER BREAK 2015 - 6 months 2018 – 6 months	Maternity leave

FELLOWSHIPS

2014 - 2016	European Marie Curie People International Incoming Fellowship (IIF) <i>University Claude Bernard Lyon (UCBL) – Lyon - France</i>
2010-2012	Natural Sciences and Engineering Research Council (NSERC) : Post-Doc Fellowship <i>Biotechnology Research Institute (IRB) - National Research Center (NRC) - Montreal – Canada</i>
2006-2009	French PhD Fellowship for Engineers - <i>Project in collaboration with Sanofi Pasteur company</i> <i>Institut National Polytechnique de Lorraine (INPL) – Nancy - France</i>

2. Teaching and administrative activities

Between 2013 and 2020, I integrated the Engineer School of Chemistry and Physics CPE-Lyon as teacher-researcher. I gained in this position several administrative responsibilities in teaching programs. I also participate to several courses either internally to this teaching institution or abroad in different type of context (foreign Master degree, WHO capacity building, Scientific society courses).

2.1. Administrative responsibility

Coordination of Specialization in Life Science & Biotechnology included in the Master of Chemistry & Chemical Engineering

CPE-Lyon engineer school – 2018 to present

I am responsible of one specialization semester in the last year of an engineering school. This program concerns between 20 to 25 students per year. This position involves training program animation (planification, teacher recruitment from academia or industry, building and improvement of teaching program, management of student evaluations), and the follow-up of trained student in their internships and career start.

Co-coordination of Specialized Mastère® in Biotechnology Process and Bioprocesses

CPE-Lyon engineer school – 2013 to present

This degree started in 2013. The first year, I was the operational coordinator to launch the formation program. I am now co-coordinator of the program with two other teachers. This program concerns professionals and post-graduate students applying for a specialization in bioprocess and biotechnology.

My activities in this program concern student recruitment and selection, planification and coordination of the scientific program on the topics of upstream and downstream bioprocesses, the recruitment of academic or industrial teachers, the co-supervision of program evolution and positioning, but also some communication activities to promote the program. I am also responsible to organize the annual scientific workshops associated to this program including 4 to 6 conferences per year. This training program concern 3 to 10 students per year.

2.2. Teaching activities

2.2.1. At CPE-Lyon engineer school

I am teaching in the 4th and 5th year of Master program in Chemistry & Chemical Engineering and in the Specialized Mastère® in Biotechnology Process and Bioprocesses. My teaching topics are aligned with the expertise I developed in my research areas.

Within these courses, several were recently adapted with pedagogic innovations to fit in the project-based learning format. This was a personal choice to propose evolutions in the teaching I launched 2 years ago. In the case of project mentoring, it also involves other teachers from different specialty that I recruited to participate to this course.

Lectures (about 34h/year)

- Animal cell culture processes and viral production processes – 9th semester
- Cellular biology – 7th semester
- Virology – 9th semester & Mastère® - *project-based learning*
- Quality by design and Process Analytical Technology (QdD & PAT)– 7th, 9th semester & Mastère® - *project-based learning*
- How to do a literature review - 9th semester
- Cell Metabolism – Mastère®

Practical courses (114 h/year)

- *Microbiology and Fermentation process – 9th semester -18 hours*
- *Biochemistry – 9th semester- 8 hours*
- *Scientific project – 7th semester - 90 hours*

Project mentoring (20 h/year) – project-based learning

- *Animal cell culture process - 9th semester - 10 hours*
- *Industrial project - Mastère® - 10 hours*

Trainees mentoring (10 students / years)

This part of my activity concerns the follow-up of undergraduate students during their final internship. It includes visiting the students and evaluating the scientific quality of their work on report and oral presentation.

2.2.2. At CPE-Lyon training center

These non-diploma courses are proposed for professionals of the biotechnology and bioprocesses industries, either technicians or engineers. Some are recurrent and take place in our training center in Lyon, some are proposed directly on industrial site. The course format are either lectures or case-studies. My teaching topics are here also aligned with the expertise I developed in my research areas.

Annual sessions

- *Theory and practice of viral production in bioreactor – 32 h/year*
- *Production and viral titration – 14 h/year*
- *In-line process analytical technologies (PAT) and Quality by Design (QbD) – 14h/year*
- *Animal cell culture process initiation – 8 h/year*

Dedicated sessions

2019 : Sartorius company - *Theory and practice of viral production in bioreactor (30 participants + 8 for practical sessions) – 20 hours*

2018 : Sanofi company (Gentilly) - *Cell culture processes and Metabolism – 8h – 7 participants*

2017 : Sanofi company (Gentilly) - *Cell culture processes and Metabolism – 8h – 5 participants*

Sanofi company (Vitry) - High cell density cell culture processes – 12h – 8 participants

2.2.3. At Scientific and Technical - IUT-A Génie Biologique – UCBL, Lyon, France

I launched in 2015 a Practical training program for Technician students. This program aims to teach cell culture processes (bioreactor handling, sterility constraint, cell seeding and growth monitoring in industrial cultivation system).

Practical courses (52 h/year) : *Cell culture in bioreactor – about 50 students /year*

2.2.4. Vaccinology and Pharmaceutical Drug Development Master, University of Siena, Italy

I was invited in 2019 to give a lecture course of 4 hours on Quality by design and PAT applied to vaccines for 25 international students coming from developing countries to be trained on Vaccinology and Vaccine production processes.

2.2.5. Cell Culture-Based Viral Vaccines Course – European Society of Animal Cell Technology (ESACT) – Llafranc, Spain

I am involved since 5 years in an international training program of 1 week supported by ESACT organization (<https://esact.org/activities/courses/>). The aim is to train international professionals and PhD students on the topics regarding the production processes of viral vaccine and viral vectors. I am part of the ten teachers of this program among with personalities of big pharmaceutical companies and representant of WHO and Bill & Melinda Gates foundation. This concerns between 20 to 30 trainees per year.

- *Principle of virology and Immunology – 1.5h/year*
- *Immunology / Virology / Cell Physiology for Vaccine Production - 1.5h/year*
- *Analytical and Potency Assays – 1.5h/year*
- *QbD and PAT applied to Viral production processes- 1.5h/year*

2.2.6. WHO Courses – Building capacities – Dakar, Senegal

I was part in 2018 of a training program launched by the WHO to build capacities in Senegal in Dakar on Viral production processes. The goal was to support the development of viral vaccine production units locally. This concerned 15 persons trained from technical staff to engineers and management.

- *Industrial virology – 1 hours*
- *Process Intensification – 1 hour*
- *Quantification and QbD – 1 hour*

2.2.7. Bioengineering Master program - McGill University – Montréal - Canada

I started to teach in the undergraduate Bioengineering master program of McGill University in June 2020. This course is entitled “Analytical assays : Potency and PAT for vaccine manufacturing” and concerns 10 students in the program of viral vectors and vaccine manufacturing specialization.

2.2.8. 3d. Fab Academy – Bioprinting program -ICBMS Laboratory – Lyon, France

This workshop is related to my new research field. The training program will be launched in November 2020 and targets to welcome 4 to 8 persons per sessions. It is dedicated to professionals who want to be trained/coached in bioprinting. It is built for 1-week lectures and hands-on sessions with a participation rate of about 40 % for my side.

2.3. Student Supervision

The supervision of PhD or Master students were in 70% of the case valorized with co-publication or oral communication.

2.3.1. PhD students: 3 students

Here are the students were official supervision as PhD director or supervisor is registered. The following section describes the unformalized supervision of student which led to co-publications.

PhD supervision as co-director

- 2020-2023 Laura CHASTAGNIER / direction planned 100% after HDR completion
1 invited communication in international conference – postponed
- 2016 – 2020 Laurent DUROUS / Co-direction 50%
 Développement d'une nouvelle technique bioanalytique basée sur l'imagerie par résonance plasmonique de surface pour le suivi en-ligne de virus influenza dans les bioprocédés vaccinaux
2 co-publications + 1 to submit / 1 communication / 2 posters in international conference

PhD supervision

- 2018-2019 Anita TOULOUÏ / 10% supervision
 Raman & capacitance spectroscopy applied to CHO cells production processes at PFIZER company.
1 co-publication / 2 invited oral communication

- 2011 Verena LOHR – Exchange and training
Perfusion based processes for influenza virus production
1 co-publication
- 2.3.2. Master degree I & II students: 10 students
- 2019 **Laura CHASTAGNIER** - Supervision 100% - Master degree Engineer school INSA Toulouse – **1 co-publication (to be submit) / 1 poster in international conference**
Lucas LEMARIE – Co-supervision 50% - Master Bio-ingénierie- Institut Supérieur des Biosciences de Paris - ÉSIPE Creteil - – **1 co-publication (to be submit) / 1 invited oral communication**
- 2018 **Olivier NICOUD** – Co-supervision 30 % - Master
- 2016 **Laurent DUROUS** – Supervision 100% - Double degree Pharmacist/engineer & Pharmacist thesis – **1 co-publication / 1 poster in international conference**
- 2014 & 2015 **Charlotte FOISSARD** – Supervision 100% - Master I & II – **1 invited oral communication**
- 2011-2012 **Christine THOMPSON** – Supervision 50% - Research Master degree - Ecole Polytechnique de Montréal – **3 co-publications / 2 posters in international conferences**
- 2012 **Alexandre LEANNERTZ** – Supervision 10% - Research Master degree - Ecole Polytechnique de Montréal – **1 co-publication**
- 2009 **Arnaud DUIGOU** - Supervision 90% - double diploma Pharmacist – Engineer School ENSIC / Nancy
- 2008 **Nicolas DENIS** – Supervision 90% - Research Master I Université Henri Pointcaré-Nancy
- 2008 **Alexandre BEGUE** – Supervision 90% - Engineer School ENSIC – Nancy

2.4. Jury Member

2.4.1. Pharmacy Thesis

- 2018 **Laurent DUROUS**
Etat de l'art des bioprocédés vaccinaux anti-influenzae et apports de la stratégie des particules pseudo-virales
- 2014 **Paul René BROU**
Culture de cellules animales dans l'industrie pharmaceutique : production de protéines thérapeutiques d'une petite échelle a une échelle industrielle.

2.4.2. PhD thesis jury

- 2020 **Daniel PAIS** – *Main examiner*
Development of advanced monitoring and control tools for rAAV production in the insect cell system.
Universidade Nova de Lisboa. Portugal.

- 2019 **Sophie BUFFIN** – *Jury member*
Evaluation des pseudo-particules grippales dans un but de vaccination par les voies muqueuses. Ecole doctorale BMIC. Université Claude Bernard Lyon 1. Sanofi Pasteur
- 2018 **Céline LOUBIERE** – *Jury Member*
“Amplification process of mesenchymal stem cells”. Ecole Polytechnique de Lorraine. Laboratory LRGP Nancy, France
Sofia CARVAHLO – *Main examiner*
“Downstream strategies for VLPs as candidate universal vaccine for Influenza”. ITQB, Portugal
- 2017 **Sonia GUTIÉRREZ GRANADOS** – *Main examiner*
“HIV virus-like particle production in CAP cells”. Universitat autònoma de barcelona escola d’enginyeria departament d’enginyeria química, biològica i ambiental programa de doctorat en biotecnologia.
- 2016 **Céline MARTIN** – *Jury member*
Etude des procédés d’amplification de cellules souches mésenchymateuses humaines. Université Polytechnique de Lorraine. Laboratory LRGP Nancy, France

3. External activities

3.1. Membership of Scientific societies & Scientific Meeting organization

I am a member of ESACT (European Society Animal Cell Technology) society since 2014. I was first selected among candidates to integrate a small group of early career scientists and engineers from academic, governmental, and private institutions. Our mission was to provide opportunities for early career scientists to personally and professionally develop whilst actively engaging in, influencing, and developing the animal cell technology community. After 5 years of activity in this group, I was recently elected by ESACT members to integrate the society executive committee in 2019. Here is part of the actions developed:

3.1.1. Organization of scientific conferences & workshops

- 2015 **1st ESACT Frontiers Retreat** - Lyon, France
115 participants - **Conference Chair** : Coordination / Budget / Sponsoring / Logistics / Social Event
- 2017 Organisation and Chair of **Workshop ESACT Frontiers at 25th ESACT Meeting**, Lausanne, Switzerland
- 2018 **2nd ESACT Frontiers Retreat**, Zagreb, Croatia
101 participants - Scientific program / Inscriptions
- 2019 **Session Chair & Organisation of Workshop ESACT Frontiers at 26th ESACT Meeting**, Copenhagen, Denmark

3.1.2. Other activities

Career & personal development workshops - I proposed and co-organized three workshops for young scientists in the context of the ESACT Frontiers Retreats.

- The first topic regarding career development aimed at providing insight in the challenges / choices / working context the young professionals could face along their career in academia, industry or start-ups.
- The second topic aimed at training young professionals to work in international collaborative environment (cultural effect on scientific interaction, personal mechanism toward team collaboration).
- The third workshop targeted the person-to-person interactions (description of personality types and their impact in a professional context).

3.2. Science popularization

Science popularization is also one of my recurrent activity. I regularly participate or propose event at the French “fete de la science” since 2013. I created an activity designed for young school kids to explain vaccine and virus productions in 2011 in Montreal. I also followed a training program about scientific journalism in Montreal in 2012-2013 in order to improve my communication skills in this field.

My latest creation of popularization activities is a Video contest proposed in ESACT bi-annual congress with a winning price to incite scientist teams to communicate on their research with non-conventional format. This was launched in 2015 and reconducted once.

4. Past and present research activities

My research activities were very much focused on the development, optimization and scale-up of viral production processes. Viral production processes are applied to both vaccine and gene therapy applications (viral vector-based products). This pharmaceutical field is in great expansion since the last twenty years because of the recurrent emergence of new viruses. The COVID-19 pandemic we are presently facing, or the Zika virus outbreak of 2015-2016 are good examples, but such processes use is also strongly pushed forward thanks to the development of new virus applications, like the oncolytic viruses evaluated for anti-cancer therapies (CART-cell therapies and lentivirus).

Thus, this field need some research support to develop new efficient, safe and controlled bioprocesses for the production of those high added-value products. It was the aim of my research activities for the last ten years. I applied my expertise and training in different research institutions in France and abroad.

Since 2009, I have worked on several viral production processes at lab- to industrial-scale levels to provide deep understanding of both cellular dynamics and viral replication kinetics with the aim to master and improve them. The Figure 1 below summarize the range of cell-based viral production processes I have been working on and Figure 2 presents the overall strategy followed.

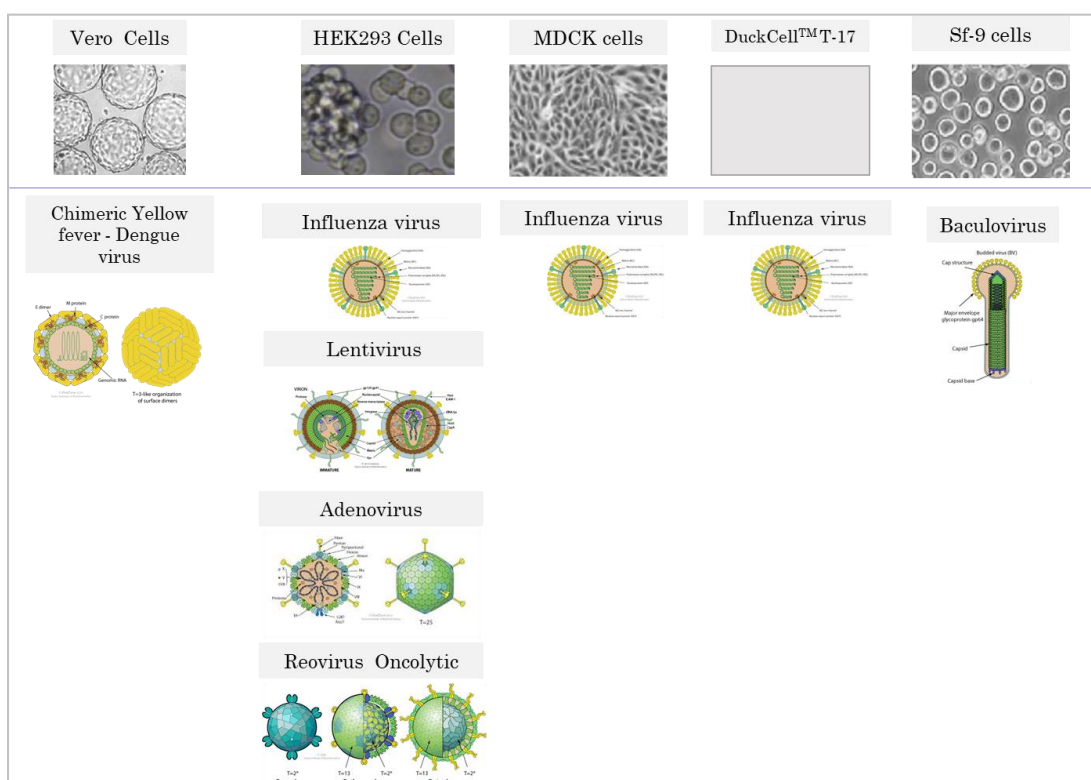


Figure 1 _ Viral production processes studied. *DuckCell™ T-17 are a proprietary cell line from transgene company. No cell line pictures are available.*

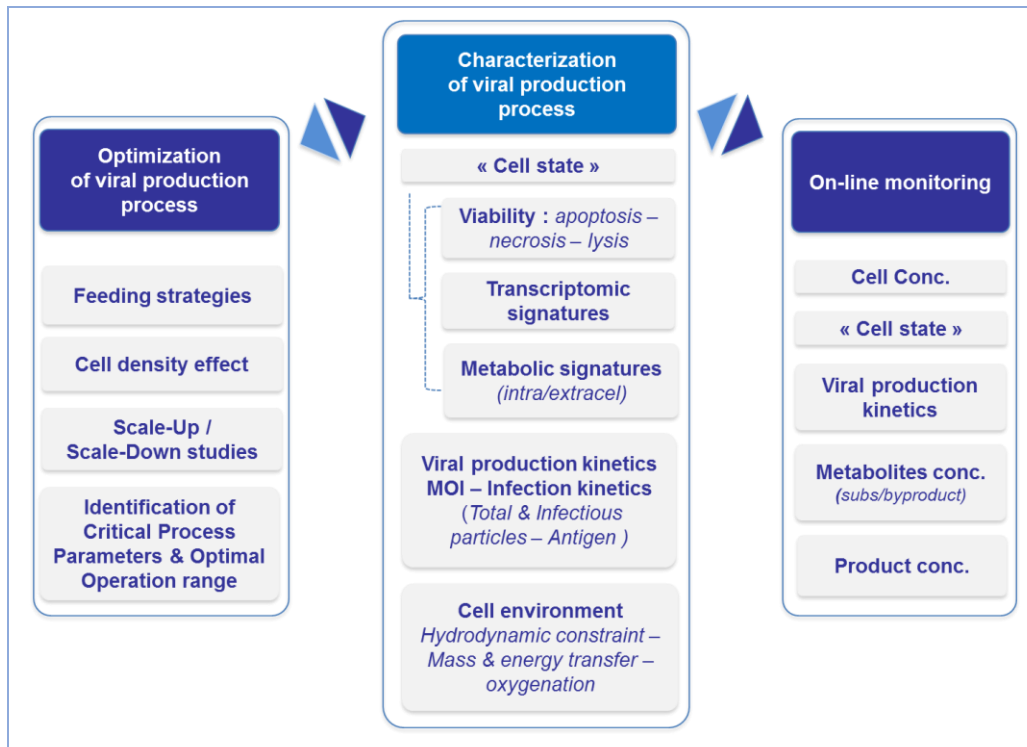


Figure 2_ Topics studied to evaluate viral production processes.

4.1. Metabolism / Viral production kinetics

On all these different viral production processes, I have applied a similar methodology to reach a deep understanding of the cellular and virological mechanisms occurring during viral replication and release. Namely, describing the biological mechanism involved (cell growth, death, metabolism, virus replication steps) in the production step and the impact of the bioprocess on these mechanisms.

Cell growth state and metabolic activity was demonstrated to have a strong influence on the viral replication kinetics. These findings were discussed for different cell platforms and virus production, in several of our or other groups' works. It is now consensually named the so-called “cell density effect” [1–3] (see section 4.1.1).

This topic corresponds to a total of 5 publications in international journals and 7 poster communications in international conferences. It also led to a 3-month training of 1 PhD student within an academic collaboration between Max Planck Institute and Biotechnology Research Institute on perfusion cultures and to 3 Master student supervision between 2006-2009.

4.1.1. Cell growth and death

Viral intracellular replication process is known to tightly regulate cell cycle, cell growth and programmed death (apoptosis) occurrence. This is now admitted by the scientific community and was described in detail in several virology studies [4–6]. Nevertheless, it is only in the past 20 years that such mechanisms were also studied combined with production process effects within the cultivation bioreactors to understand cell growth and death pattern in a cell-based production [1,7].

Thus, in one of our study we described the impact of cell culture feeding (batch versus perfusion feeding) on the growth and death pattern of a HEK293 cell culture infected by influenza H1N1 PR8 virus. As expected, the levels of cell death by necrosis and apoptosis were much lower with perfusion continuous feeding, allowing to maintain high cell viability prior infection (see Figure 3). Cell death pattern occurring just after infection is specific to the type of virus infection. In the case of influenza virus, a reduction of dead cell populations (apoptosis and necrosis) is commonly observed during the first 24 hours post-infection [7,8], namely the first infection cycles (see section 4.1.3). This was later explained by studies demonstrating virus-driven interaction between few of its viral proteins and key host-cell factors regulating cell cycle and apoptosis pathways (see section 4.1.3). For example NS1 is known to interact with p53 and AKT [9–11] to promote cell survival in first replication phases and then with HSP90 and alpha tubulin to enhance apoptosis in late replication phases [10].

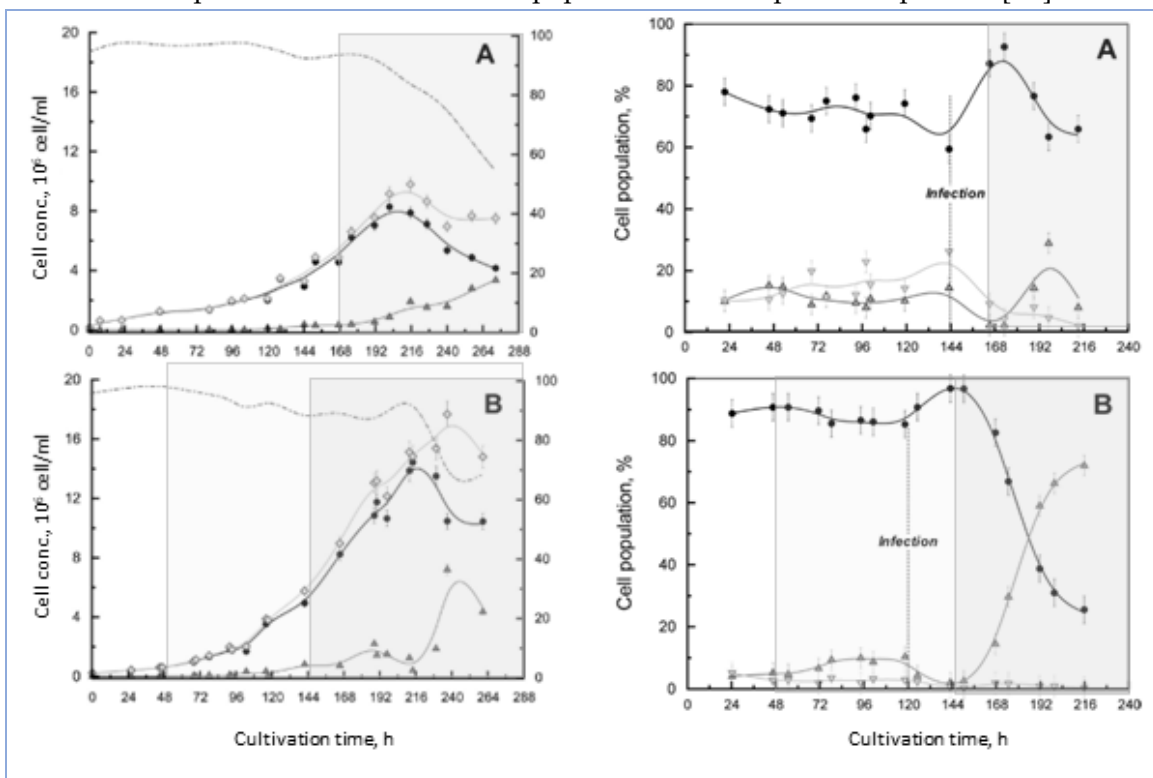


Figure 3 _ Cell growth and death pattern comparison for influenza infected cultures of HEK293 S cells. A : Batch culture. B : Perfusion culture. Reproduced and adapted from Petiot et al. 2011 [1].

“Cell density effect” (CDE) was first identified in 1994 on Sf-9 cells for the production of β -galactosidase following baculovirus transduction [12]. It was later extensively described for HEK293 cell platform as reviewed in one of our publication [13] and is now discussed for most of the cell-based production processes [3,14–16]. This cellular mechanism was identified specifically in cell-based viral production processes since in that case the target is to maximize the viral production levels. Nevertheless, the generalized practice in virology protocols to infect cell layer at specific medium confluency rates most probably uses the same concept.

CDE corresponds in “a decrease in virus per cell productivity appearing concomitantly with the increase in cell concentration at infection” [13] (see Figure 4). CDE is commonly observed at cell concentrations of $1\text{--}2 \times 10^6$ cells/ml, whereas standard cultivation processes with optimized media could easily reach cell concentration over 10×10^6 cells/ml. CDE was demonstrated to be cell line, virus and medium-dependent. Thus, HEK293 cells demonstrate to have a CDE at $1\text{--}2 \times 10^6$ cells/ml for adenovirus production and at 4×10^6 cells/ml for influenza production. Thus, such production limitation is strongly related to the cell physiology all along the process and to as well as the viral replication mechanism and interaction with the host cells. Consequently, media composition, feeding strategy and their associated impact on cell metabolism were demonstrated to be strongly related to CDE.

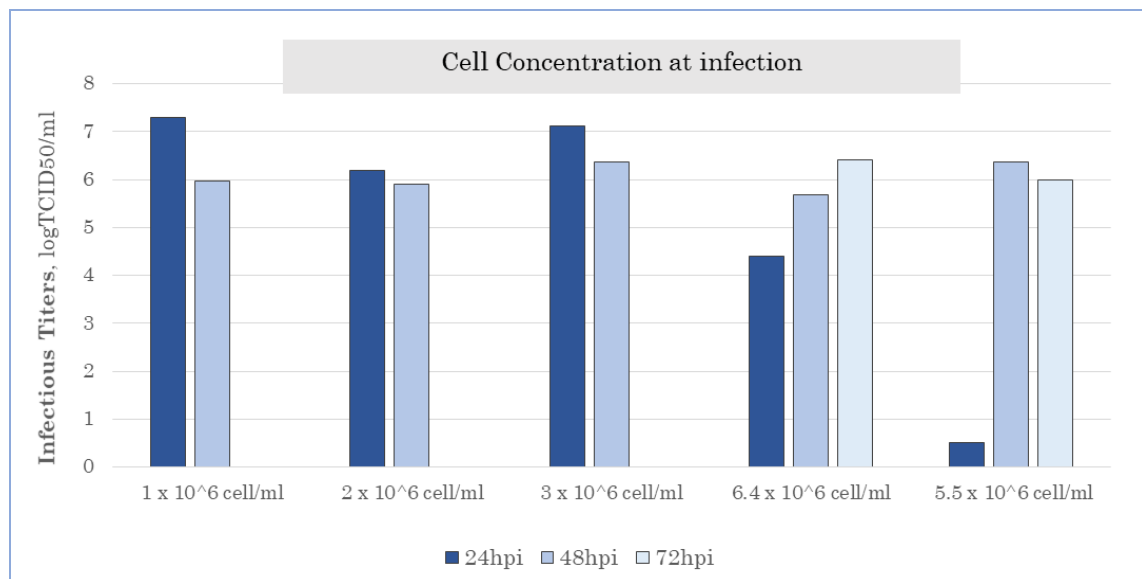


Figure 4_ Viral production kinetics of infectious particles for influenza productions produced in batch cultures. Adapted from Petiot et al. 2017 [3].

4.1.2. Cell metabolism

For all the cell-virus systems I had to work with, deep characterization of cellular metabolism was performed including monitoring of extracellular and intracellular metabolites. Standard metabolic characterization generally relies on the sole monitoring of extracellular molecules; the two main nutrients (glucose and glutamine) and the inhibitor co-products issued from their metabolic degradation (lactic acid and ammonia respectively). Here, characterization was systematically including amino

acids monitoring [1–3]. Additionally, development of methodologies to quantify intracellular metabolites were developed and allowed to access to amino acids pools as well as to accumulated carboxylic acids of Krebs cycle ([2] and unpublished data HEK293-influenza virus 2011– see Figure 5).

This approach allowed to identify the metabolic pathways behavior under different feeding strategies and to propose novel hypothesis concerning links between cell feeding, viral productivity and the so-called “cell density effect”. While for a long time researcher focused on feeding strategies to avoid nutrient limitation, our data allowing a deep understanding of cell intracellular metabolic state in HEK293, thanks to NMR quantification, demonstrate that CDE involving virus production reduction was due more to inhibitory effect than to nutritive limitation. In this study, we demonstrated a high accumulation of acids issued from amino-acid degradation pathways within the cells (formic acid, 3-methyl-2-oxovalerate, 2-oxo-isocaproate) while cells reach high cell density thanks to supplemented media (see Figure 5).

This inhibitory effect was early demonstrated by a media component screening using Design of Experiment (DOE) with fractional factorial design to select best serum free media composition for VERO cell growth [17]. In this study, addition of the media part composed of the 20 amino acids strongly impacted cell growth with a half diminution of the maximal cell density reached. Since then, several new studies on protein production at large-scale on CHO cells demonstrated similar mechanism and confirmed our hypothesis [18].

These works demonstrate that applying alternative feeding strategies, like fed-batch with media exchanges in the case of Vero cells, or perfusion systems in the case of HEK293 cells (see Table_1) allowed to overcome cell metabolic inhibiting phases. It also allows to maintain balanced and higher metabolic activities within the central carbon metabolic pathway over the viral production phase [1]. They commonly resulted in improvements on cell growth either on cell growth phase or on cell viral production phase.

Viral production type	Cell density at infection	Feeding strategy	Improvement rate	References
Adenovirus	2×10^6 cell/ml	Batch		[1,70,71]
Adenovirus	1×10^6 cell/ml			
Adenovirus	8×10^6 cell/ml	Perfusion: 0.2 and 1.2 vol/day	4.3-fold vs batch	[57,71,89]
Adenovirus	11.8×10^6 cell/ml		8.8-fold vs batch	
Adenovirus	1×10^6 cell/ml	Medium exchange	1.6-fold vs batch	[50]
Adenovirus	0.5×10^6 cell/ml	Fed-Batch with medium supplement: Cell boost 5	5.4-fold vs batch	[78]
		Foetal bovine serum*		
Influenza	4×10^6 cell/ml	Batch		[58,64]
Influenza	6×10^6 cell/ml	Perfusion: 0.5 vol/day	3.5-fold vs batch	[64]
Lentivirus	8×10^6 cell/ml	Perfusion 2 vol/day +medium supplement Cell Boost 5	26-fold vs batch + perfusion started at 48 h post-transfection	[82]
HIV gag VLPs	$2-3 \times 10^6$ cell/ml	Medium exchange + Lipid mixture supplement	7-fold vs batch	[79]
Retroviral vectors	4.5×10^5 cell/ml	Fed-Batch with medium supplement: pluronic, lipid mixture Foetal bovine serum albumin*		[90,91]

Table 1_ Examples of viral production performed in HEK293 suspension cells. All these productions were performed in serum free culture conditions except *. Reproduced from Petiot et al. 2015 [13].

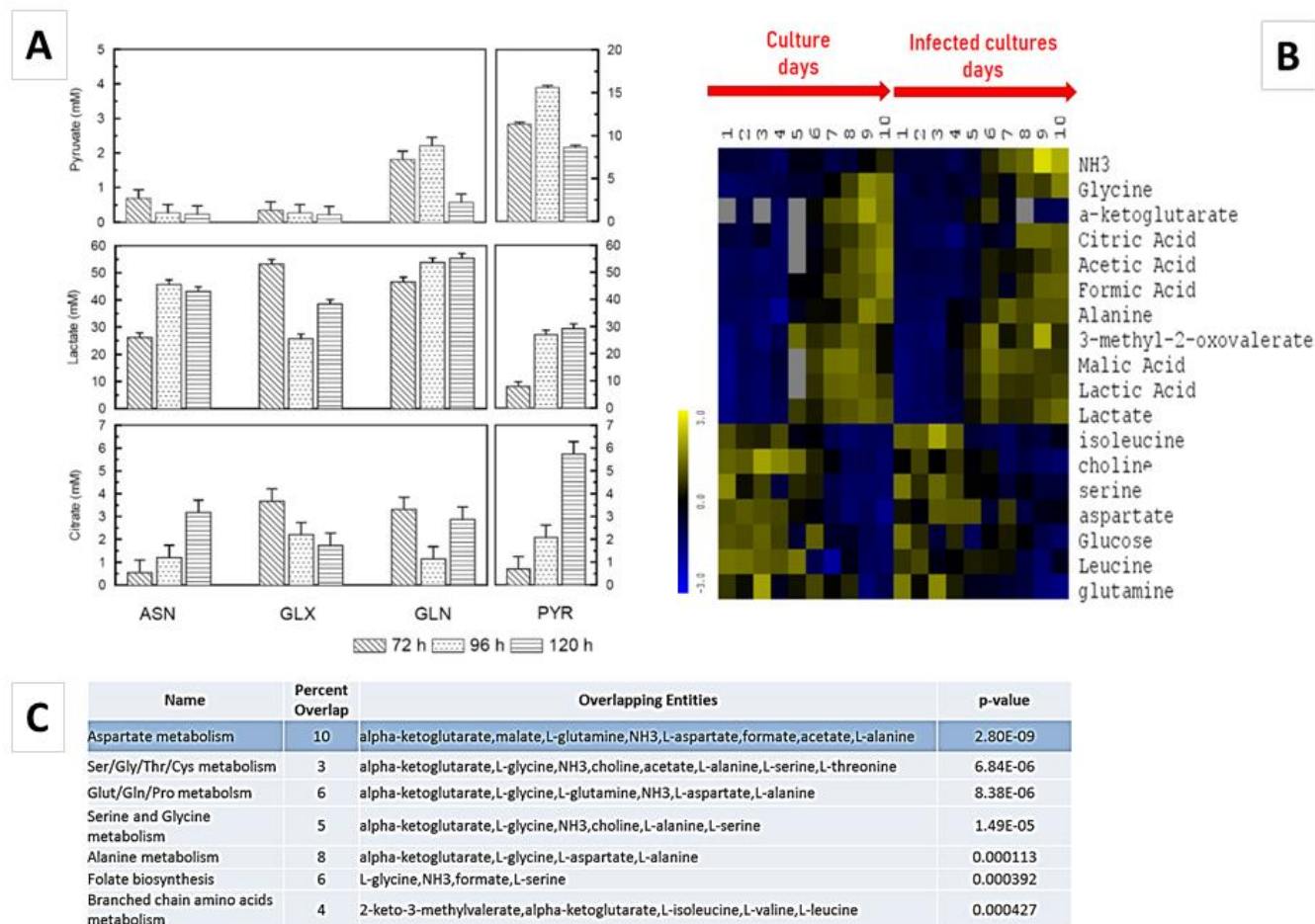


Figure 5_ Accumulated intracellular inhibitory metabolites. A. HPLC analysis of intracellular metabolites accumulated during Vero cell growth (Adapted from Petiot et al. 2010 [2]). B. NMR analyses of extracellular metabolites during infected and non-infected cultures of HEK293 cells (non-published data). C. Kegg database analysis of metabolic pathways involving accumulated metabolites (non-published data).

Further applied to productivity level, such strategy was demonstrated to be highly efficient to produce dengue chimeric virus on Vero cells. Both time of harvest, and production level were improved with an adaptation of the media composition and the substitution of glutamine by other carbon sources. The most efficient one was the dipeptide alanine-glutamine, also known commercially as Glutamax® and which use was first described by Christie et al. 1994 [19].

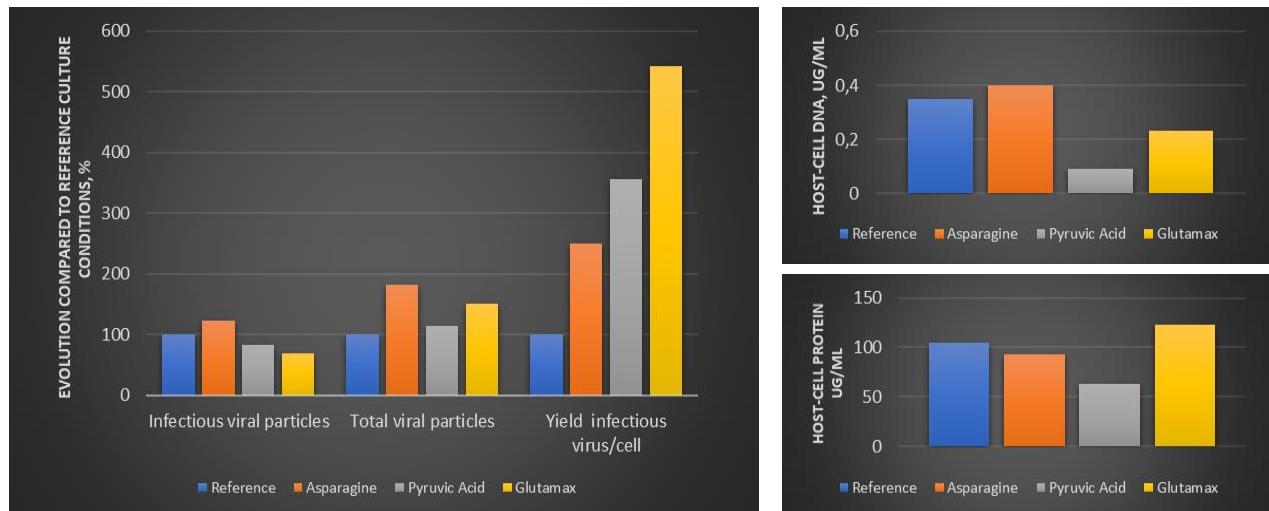


Figure 6_ Impact of metabolic activity on production levels and product quality. Adapted from PhD manuscript entitled « Vero cell culture processes in serum-free medium: Contributions to Process Analytical Technology approach ».

Nowadays such cultivation strategy is extremely widespread over cell culture practice, as shelf long term storage media formulation are proposed with Glutamax® feed to support glutamine feeding. Such media are proposed for all type of cell cultures such as production cell lines, primary cells, and stem and iPS cells. Further studies to link metabolic pathways over-saturation and viral replication phases are needed and currently explored by different teams.

4.1.3. Virus replication kinetics

I had to work with multiple cell-virus couples to produce viral or antigen stocks (see Figure 3). The virus replication mechanisms and the process impact on these mechanisms are often poorly understood. Therefore, several of my research projects investigated the virus replication process and timing. Part of these studies were published in international journals or documented in my PhD manuscript, but some was not considering the confidential agreement with some partners.

The most deeply studied model I had to work with was influenza virus produced in HEK293 suspension cells. Here, complete replication phases were described over process courses and linked with process parameter impacts. Thus, “Multiplicity of Infection” (MOI) levels were demonstrated to influence the final production titers as well as time to harvest. This study allowed to visualize the several infection cycles within the production process and to demonstrate that maintaining cell growth during the infection process is a key part for higher production rates. It is also one of the few

studies allowing to clearly identify the duration of a viral replication cycle within a production process.

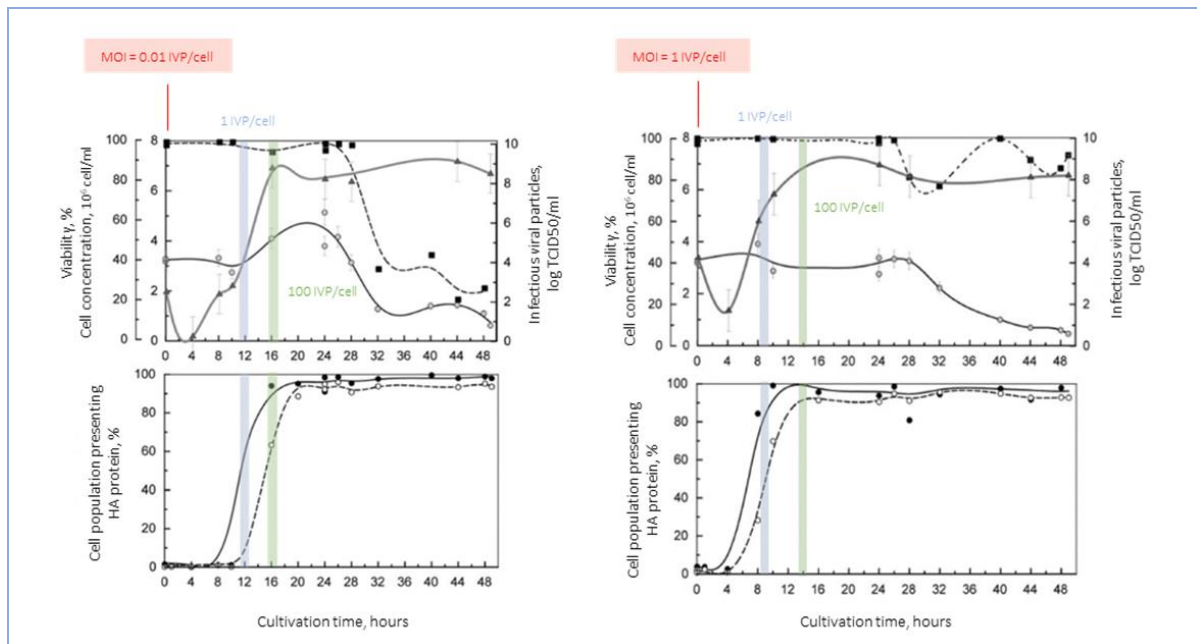


Figure 7_ Influenza replication kinetics in HEK293 cell cultivation process.
Adapted from Petiot et al. 2012 [20]

Impact of cell line and viral cell strain were also investigated [21,22]. This confirmed that the replication ability and kinetic of a viral strain are strongly dependent of the cell-host. Indeed, in a later published paper describing replication kinetics of influenza viruses on avian suspension cell line, the replication process was much faster than in the human HEK293 cells. The replication kinetics was also demonstrated to be strongly dependent of the strain-host interactions. Indeed, in the case of influenza it is known that permissiveness of an influenza virus toward a production cell line is linked to the presence, the type and the repartition of sialic acids (α -2,3 or α -2,6) present on cell receptors in respiratory tract [23].

Trying to deeply understand the viral replication cycles and their impact on cellular behavior, obviously lead to the glaring lack of satisfactory analytical methods to characterize the biological events occurring in the production phases. Therefore, other viral infection cycles will be further discussed in analytic subsection where development of new technologies allowed to further understand viral replication kinetics.

4.2. Development of analytics dedicated to viral production processes

Viral production processes strongly lack efficient tools to better understand, monitor and finally control the biological processes during the productions. This was extensively reviewed in one of our work on influenza-VLP processes where the analytical needs are even more pressing [24]. Therefore, this is major part of my research activities over the

past 10 years. The development of quantification, characterization or methodologic tools were applied to the different viral production processes I was studying. I had a strong focus to implement technologies allowing to perform “in-process” measurement. Indeed, the final goal of such technologies would be to allow for process supervision and later feed-back control on the production processes. These research were strongly related to the Quality by Design (QBD) and Process Analytical Technology (PAT) guidance published in 2004 by FDA for pharmaceutical companies [25].

Thus I focused on the three main targets of a viral cell-based production process namely the productive cell populations, their metabolic state and ultimately the viral product (antigen or total viral population productions) (Figure 8).

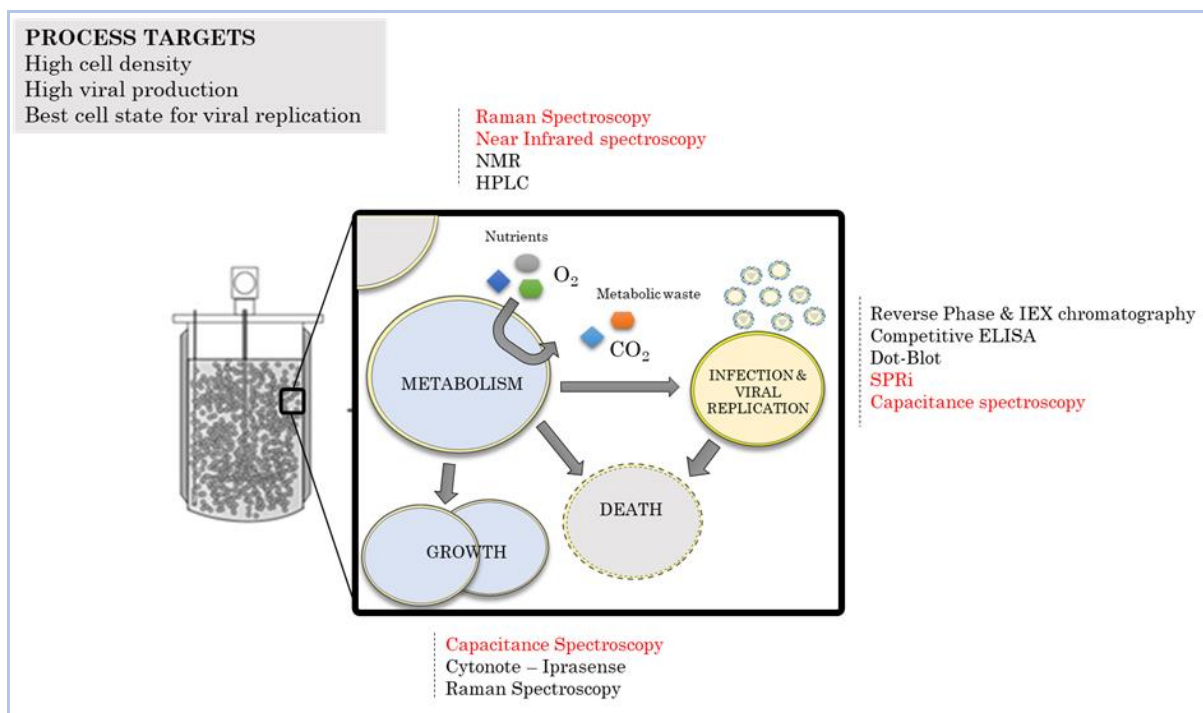


Figure 8_ Technologies developed, implemented, or evaluated to reach in-process and in real-time monitoring of viral production processes. In red are labeled the technologies implemented in-line.

The developed expertise in this field and the renown associated with the technologies presented in the following sections led to several subcontracting for data analysis or co-development with industrial companies (PFIZER Inc, Medicago, Oncolytics).

These works allowed to 11 papers published in international journals, to 8 oral communications and to posters in international conferences. This research field also allowed for the supervision of 1 PhD student as co-director, 1 PhD student in an industrial collaboration and 3 Master students.

4.2.1. Monitoring of cell growth and death

To fulfill needs of more advanced solutions to fasten, standardize, and improve the growth monitoring in animal cell-based culture processes and answer to the FDA QbD guidance, several technologies were evaluated in the past 20 years. In my case, one of the first analytical development performed was applied to anchorage-dependent cells and concerned the set-up of an off-line imaging technology to analyze cell surface coverage in 96-well plates for media screening and serum-free medium development [17].

Nevertheless, cell culture-based processes would better benefit from the implementation of on-line monitoring tools for cell biomass. To date, cell-based processes primarily rely on routine off-line sampling and analysis for characterization and supervision. Trypan blue staining remains the industry standard to measure cell concentration. This method quantifies viable cell numbers by manual or automated numeration with dye exclusion enabling to discriminate between viable and porous dead cells. This approach has the intrinsic disadvantage that response times to process events and variations are delayed and are performed “based on the last sample that was analyzed”. Rational control, i.e. direct process-event-based, is consequently challenging with sampling frequencies ranging from hours (best case) to days (worst case). The timing and execution of time-critical process actions such as the addition of nutrient feed, the induction of production phase or the onset of product harvest are still mainly based on accumulated historical data on the production system, on operator experience or simply performed in a time-based manner. On the other hand, analytical workload increases when high throughput scale-down reactor models are used for process condition screening.

Second, the trend towards continuous bioprocessing to reduce manufacturing costs creates additional needs for advanced process control strategies and hence the implementation of “meaningful” bioprocess sensors. Thus, on-line techniques such as permittivity probes provide results faster and cheaper. Therefore, an increased amount of industrial processes is now equipped with capacitance biomass probes for cell growth monitoring.

Our works applying the capacitance technology to viral production processes with both adherent and suspension cells were among the precursor in the field in 2006 [20,26,27] (discussed in section 4.2.3). There is now a significant number of published results available that illustrate how the technology monitors changes at the cellular level that are related to the physiological state of the cell culture, and can hence be exploited to control and trigger nutrient feed additions, detect virus release and critical process events (apoptosis, viability, cell size).

The technology allows for the on-line monitoring of the biovolume of cell present in an analytic volume of the cell culture bioreactor. A non-negligible advantage of the technology concerns its application to cell-based process using microcarrier bead for anchorage-dependent cells. Indeed, standard cell counting is commonly marked with significant errors due to the difficulties to detach the adhered cells. In one of our

application of capacitance probes, such probes were proven to be a valuable tool to improve the quality of the Vero cell growth monitoring [26].

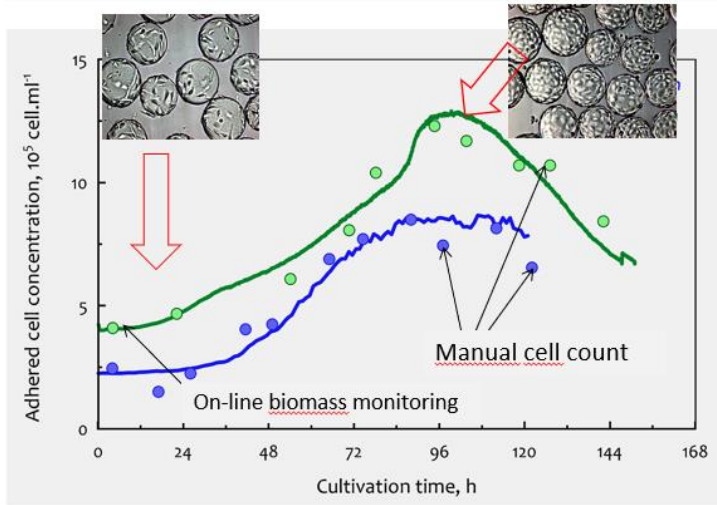


Figure 9_ On-line monitoring of adherent Vero cell grown on microcarrier. Adapted from Petiot et al. 2012 [26].

The second strategic analytic breakthrough positioned in this study, and later reproduced for several suspension cell lines [28–30], was the on-line detection of apoptosis onset in adherent cell culture processes [26] (see Figure 10).

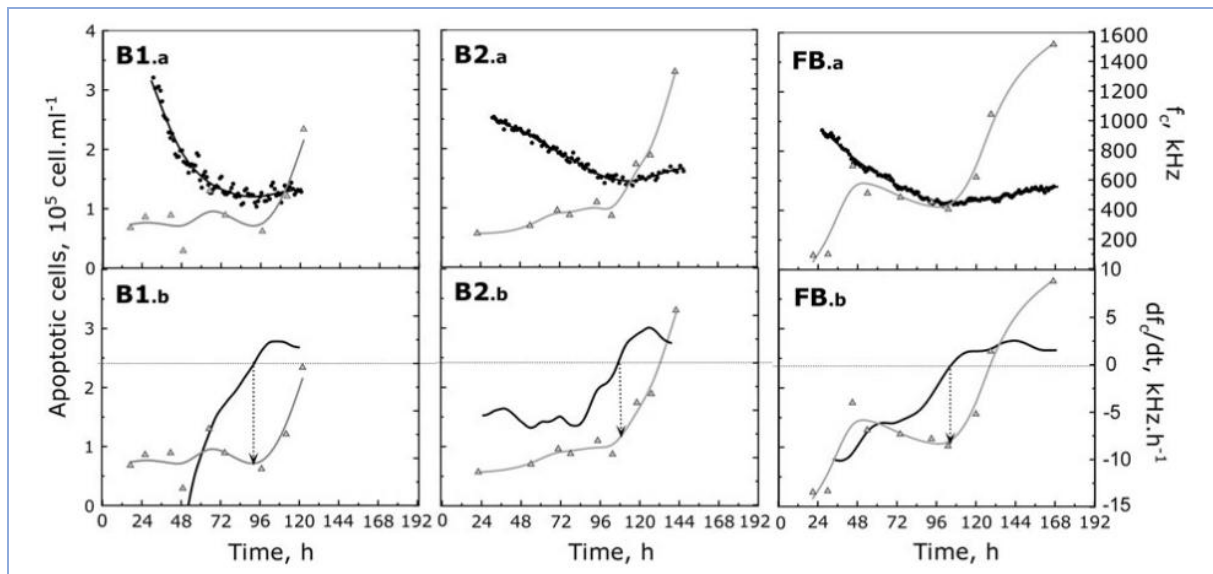


Figure 10_ Detection of Vero cell apoptosis with capacitance probe. Evolution with time of apoptotic cell concentration (grey triangle) opposed to characteristic frequency (f_c) parameter and its derivative df_c/dt (black lines). Compared trends for batch cultures (B1, B2) or fed-batch culture (FB).

This study allowed to demonstrate that early detection of cell death pattern in bioproduction thanks to mathematical signals treatment was possible and could further allow for early detection of strategic biological or process event (i.e. viral production start, abnormal process related cell-death).

4.2.2. Monitoring of cell metabolism

As demonstrated in section 4.1.1 & 4.1.2, cell metabolism understanding is also of strategic importance for cell-based and viral production processes. Therefore, along the past years, a strong focus was put on implementing on-line monitoring strategy for released inhibitor metabolites or strategic consumed substrates. Two technologies were evaluated in my work, both based on spectroscopic principles, Raman and Near Infrared Spectroscopy.

The principle of the application of such technology to cell media composition monitoring rely on the real-time acquisition of a media fingerprint in spectra which will be further analyzed using chemometrics. The chemometric analysis aims at extracting specifically information about the targeted metabolites from the spectra acquired which represent the overall sum of spectrum of all media components. Thus, chemometric analysis and building of dataset to produce robust and valid calibration here a critical steps of the method (see Figure 11 for description of the steps). Such experiments often necessitate datasets > 100 samples which are prepared by media mixing plan and robust Design of Experiment (DOE). This method was explored for two processes of my research projects, one in order to monitor the four main metabolites (glucose, lactate, glutamine and ammonia) of a Vero cell culture process by NIRS spectroscopy, and the second to reach three metabolic inhibitors (2-methyl butyrate, iso-butyrate, isovalerate) which are only in standard pilot-scale production by costly NMR quantifications and which have been monitored thanks to Raman Spectroscopy. This last project was a collaboration for method co-development with Pfizer St Louis US company.

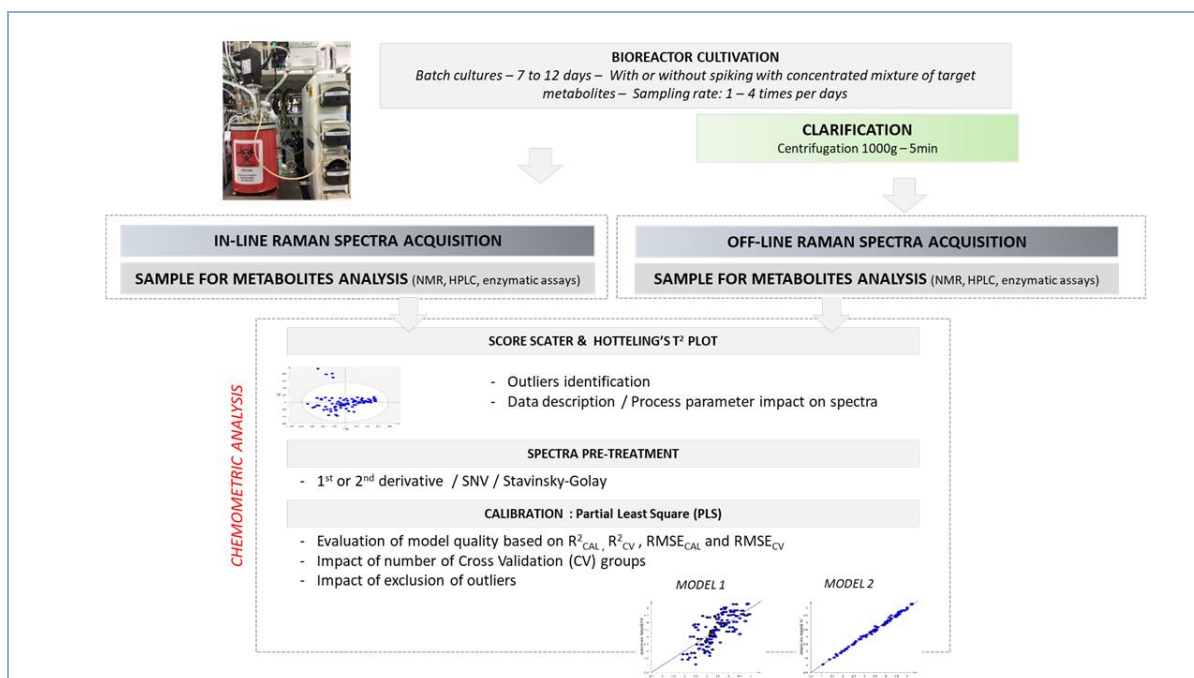


Figure 11_ Calibration building from chemometric analysis of Raman or NIR spectroscopies for metabolites monitoring.

Near Infrared Spectroscopy (NIRS)

Application of NIRS to adherent cell based cultivation using microcarrier beads necessitated the development of a dedicated NIRS probe with variable optical path [31]. This was a co-development with Thermofisher Inc. company allowing to propose a probe solution for microcarrier based cultures. A complete dataset of 95 samples was necessary to reach satisfactory calibration for two metabolites over the four targeted (glucose & lactic acid). This study was in 2010 among the first to exploit spectroscopic analyses to monitor bioreactor cell culture. It is now recognized that low concentrated metabolites, like glutamine and ammonia, in this study are complex to reach and necessitate more samples (over 200). The specificity of our study was to integrate spiked samples in the dataset to decorrelate metabolic pathways and ensure the proper calibration towards targeted metabolites.

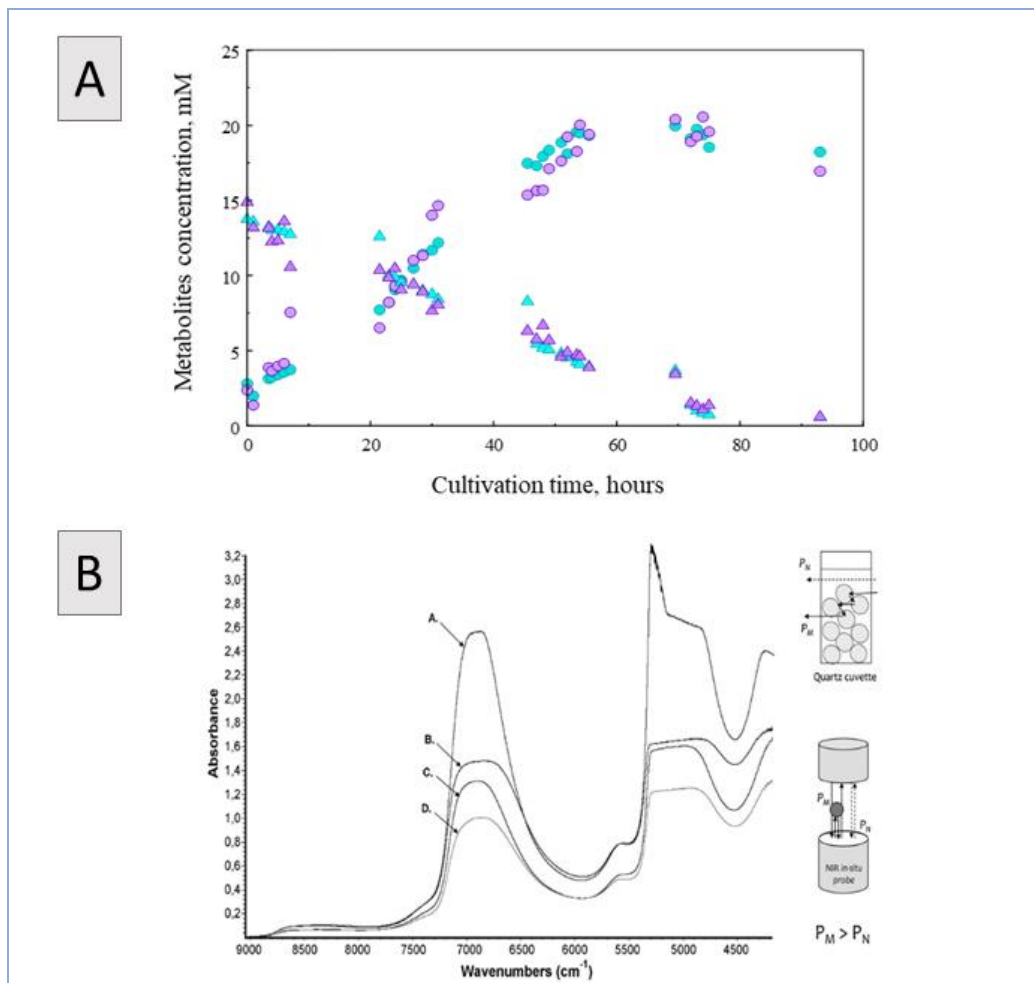


Figure 12_ A: Glucose (▲) and Lactate (●) monitoring by NIRS (▲, ●) compared to reference enzymatic quantification (▲, ●)- B : Spectra acquired off-line and on-line for NIRS applied in the case of microcarrier based cell culture (A&C- Media containing microcarrier, B & D- Media without microcarrier). Adapted from Petiot et al. [31].

Raman spectroscopy

A similar methodology was recently co-developed with Pfizer US company and currently under review at *Biotech and Bioengineering* journal to on-line monitor metabolic inhibitors produced in CHO cell Culture processes. The targeted metabolites were here low concentrated metabolites which could not be monitored with standard or advanced on-line monitoring currently available on bioprocesses. The only mean to access quantification of these three metabolic inhibitors was thanks to off-line, costly NMR assays. Thus, the implementation of an on-line tool to access levels of production and cell growth inhibitor in real-time is of great interest for industry and academia developing new bioprocesses. Although this work is applied to CHO cell cultivation, its applicability to viral production processes is direct as we also identified similar metabolic inhibitors for viral production most probably at the origin of the so-called “Cell Density Effect” previously described (see section 4.1.117). DOE building and systematic evaluation of spectra pre-treatment is essential to reach reliable calibration and quantification methods (see Table 2).

This work allowed us to propose quantification for 5 over the 6 metabolites targeted. In the present case, correlation and prediction performances are lower than for highly concentrated metabolites like glucose and lactate, but still sufficient for *on-line* detection of inhibitors accumulation or amino acids depletion.

The second major finding of this work was the strong impact of cell composition on the calibration process. The example of isoleucine presented in Figure 13 demonstrates that, in this case, intracellular content of the cells in isoleucine hinder the correct quantification of extracellular isoleucine. This observation is interesting to link to the fact that both Raman and NIR spectroscopy are efficient tools also to implement cell biomass monitoring.

Table 2 _ Prediction models and quantification performances thanks to Raman spectroscopy. 1st & 2nd derivative (1st deriv; 2nd deriv), Stavinsky-Golay (SG) and Standard Normal Variate (SNV) spectral pre-treatment were applied.

Metabolites	Sample Dataset	R ² _{PRED}	RMSE _{PRED} , mM	% Conc. Range	Spectra pre-treatment
2 Methyl butyrate	145	0.73	1.07	15%	1st deriv + SNV
Isobutyrate	149	0.58	1.10	55%	1st derivative
Isovalerate	149	0.72	2.23	49%	1st deriv + SG
Leucine	145	0.82	1.46	21%	2 nd derv + SNV
Valine	147	0.80	1.34	19%	1st deriv

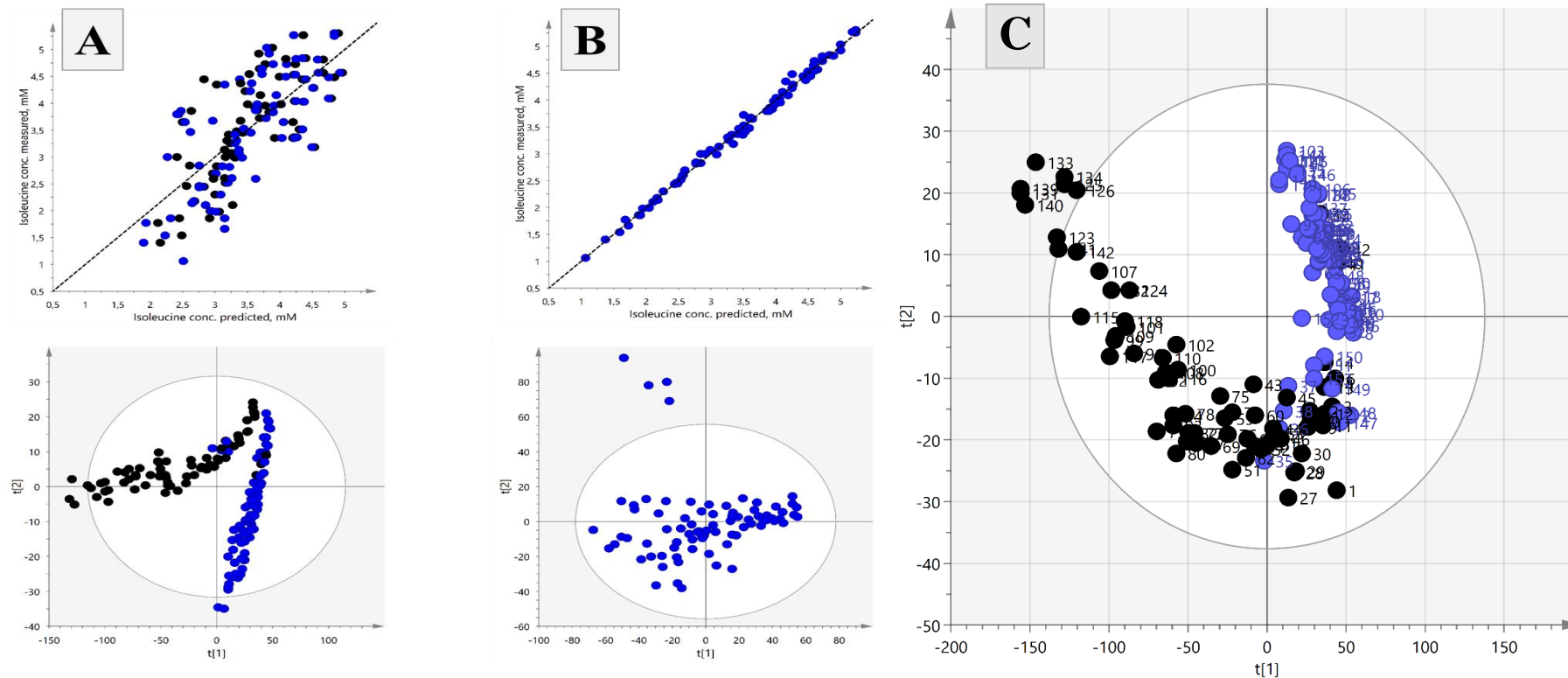


Figure 13 Off-line calibration models for Isoleucine. A- Score scatter plot and calibration curve for PLS model including all the samples of the calibration sample set (153 samples – UV scaling – 2 PLS components – R^2_{CAL} 0.522 – R^2_{CV} 0.475 – $RMSE_{CAL}$ 0.71mM – $RMSE_{CV}$ 0.74 mM). B- Score scatter plot and calibration curve for PLS model including only clarified samples without cells of the calibration sample set (80 samples – UV scaling – 9 PLS components – R^2_{CAL} 0.994 – R^2_{CV} 0.812 – $RMSE_{CAL}$ 0.08mM – $RMSE_{CV}$ 0.75 mM). C- Score scatter plots for PCA model describing the DOE off-line calibration sample set. Impact of presence of cells in the samples on principal component $t[1]$ and $t[2]$ PCA model components. ● non-clarified samples with cells; ● clarified samples without cell. Adapted from Petiot et al. *Biotech Bioeng.* Under review.

4.2.3. Monitoring of viral production kinetics and production levels

In viral production processes, the holy grail for manufacturers would be to have a direct access along the production steps to the quantification and qualification of the product. For other productions it is now possible to have an easy quantification of the end-product as for MAbs production for example. In that case, easy and rapid off-line or at-line quantification tools ranging from ELISA assays, to Ion Exchange Chromatography (IEX) chromatography [32] or on-line Raman applications [33,34] are available. In the case of viral quantification, scientific community is still stuck with old-fashioned, highly variable, and cost-intensive *off-line* assays (pfu or TCID₅₀ to quantify infectious viral particles, and Single Radial Immunodiffusion assay (SRID) or hemagglutination for antigen or viral protein content). Another main critical analytical challenge of influenza vaccine lot quantification is the use of reference serum and antigens in the SRID assay which necessitate about 4 months to be produced annually. This assay strongly impedes the development of new vaccination strategies and of quick response to pandemic outbreak. **Therefore, to develop efficient new processes, I have focused part of my research on developing assays allowing for a rapid, specific quantification of the viral particles.**

Reverse-Phase and ion-exchange Chromatography

Reverse-phase and ion-exchange chromatography (IEX-HPLC) are the first two methods that have been evaluated for viral quantification. IEX-HPLC has already been applied for the quantification of several virus (adenovirus, AAV, Baculovirus), and the present study consists in applying this technology to influenza virus, VLPs and lentivirus [35]. Main advantage of such technology is that it allows for analysis of various sample preparations from crude samples extracted from bioreactor cultures up to highly purified materials. Main result here was that we were able to achieve, for several influenza strains, satisfactory correlations between counts performed on electron microscopy grids, which is a laborious and long-lasting technique, with HPLC peaks. However, a differential response in intensity was observed for the different strains tested.

This last observation hinders to propose a generic correlation to quantify all strains with a single protocol. Here the differences between strains in glycoprotein density on the viral surface, in amino acid composition or on particles morphology (ex. filamentous forms of B strains) could account for these changes [36].

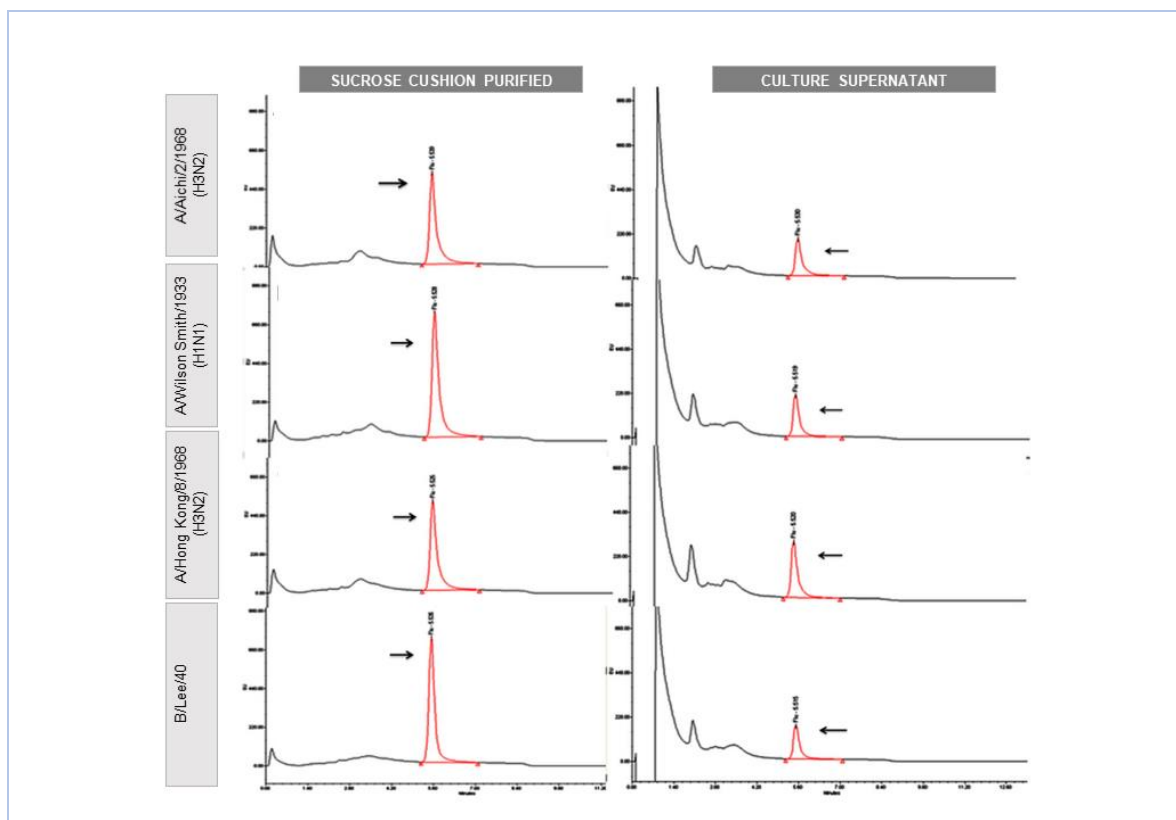


Figure 14_ **HPLC profiles of sucrose cushion purified influenza viruses;** (A) A/Aichi/2/1968 (H3N2), (B) A/Wilson Smith/1933 (H1N1), (C) A/Hong Kong/8/1968 (H3N2) and (D) B/Lee/40 and virus supernatants; (E) A/Aichi/2/1968 (H3N2), (F) A/Wilson Smith/1933 (H1N1), (G) A/Hong Kong/8/1968 (H3N2) and (H) B/Lee/40. The virus peak is indicated by an arrow. Extracted from *Transfiguracion et al.* [37]

ELISA and Dot-Blot assays

An alternative strategy to reach universal quantification was proposed while developing universal antibodies against HA protein [38]. These antibodies were generated against a portion of a highly conserved region of hemagglutinin, called the “hemagglutinin fusion peptide” to allow for a generic recognition of the influenza strains A and B. The aim was here to directly replace the Single Radial Immunodiffusion assay (SRID) strongly limiting the production of annual and pandemic vaccines, as well as the hemagglutination assay which is a highly variable assay. Two quantification protocols were proposed, Dot-Blot and competitive ELISA assays.

Preliminary evaluation by immunoblotting allowed to demonstrate that nearly 40 strains of influenza with subtypes H1 to H13, as well as B strains from both the Yamagata and Victoria lineage were detected by the pan-HA cocktail. Also, the panel of samples tested include recombinant HA proteins, viruses produced in eggs and mammalian cells but also influenza-VLPs prepared in tobacco plants (Figure 15).

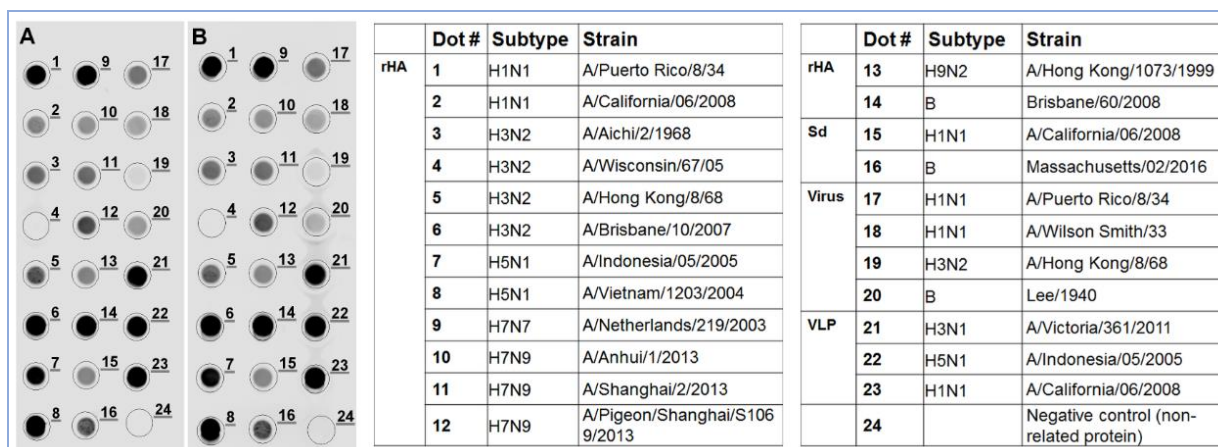


Figure 15 _ Dot blot results with a pan-HA cocktail of antibodies obtained from mouse hybridomas (A) and CHO produced pools (B). Each dot represents a different strain as indicated in the table on the right panel. Legend : rHA = recombinant hemagglutinin; Sd = cell-produced calibrating standards (NIBSC); Virus = viruses produced hemagglutinin; VLP = Virus like particles produced in plants (Medicago Inc). Extracted from Manceur et al. [38].

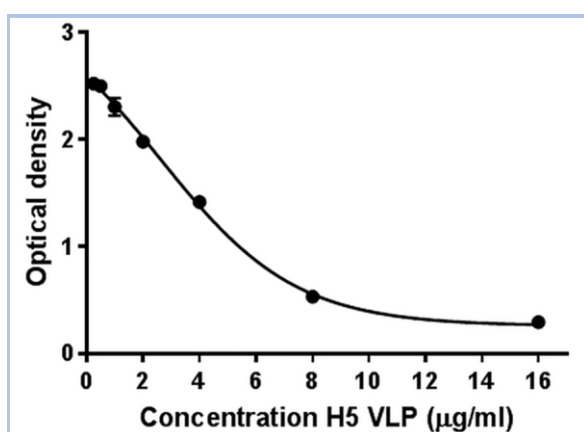


Figure 16 _ A standard curve was generated with H5 VLP from 0.25 to 16 µg/ml in a competitive ELISA. Extracted from Manceur et al. [38].

Correlations between Dot-Blot and standard assay (SRID or hemagglutination assays) were satisfactory although not robust enough (Hemagglutination assay: $R^2 = 0.80$ and SDV = 4.5% ; SRID : SDV 13-23%). One advantage of this strategy is that HA measured by dot blot includes monomers, trimers and oligomers whereas only trimeric HA is measured with the SRID assay.

The main drawback of such strategy is the affinity discrepancy of the universal antibodies generated towards the various influenza strains. Results showed that mAb 11H12 binds preferentially to influenza group 1 strains, and mAb 10A9 binds preferentially to influenza group 2 strains. The consequence being here that a screening and a specific calibration with known concentrations of viral material will be needed annually as for standard assay currently in-use. At that stage, such approach, although it allows for quantification with lower detection level suitable for VLPs strategies (250ng/ml) (Figure 16), does not reach the initial goal to replace the standard assays and to get rid of calibrating reference antigens. Additionally, bioactive hemagglutinin or bioactive viral particles could not be quantified with such strategy as these assays necessitate in their

protocol a 4M urea denaturation step. Indeed, it allows to denature hemagglutinin to access its “fusion peptide”. Therefore, other quantification strategies were later explored.

Surface plasmon Resonance imaging

Surface plasmon resonance imaging offers interesting capabilities for the quantification of influenza viruses. It was recently proposed to replace SRID assays in the context of pandemic outbreak to speed up the vaccine lot release. Assay principle holds on a biochemical interaction between the protein (antigen) and a ligand grafted on the biosensor chip surface (antibodies, aptamers, nucleic acids, cell-receptors). Such assays are commonly fast, real-time, and allow for detection of virus at low concentration levels [39].

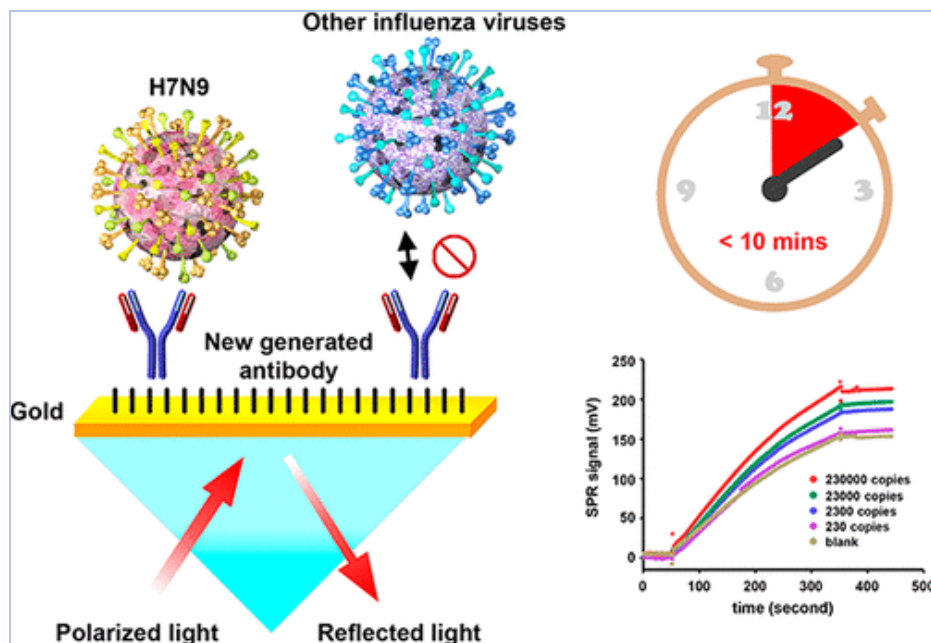


Figure 17_ Surface Plasmon Resonance application to influenza virus detection.

Extracted from Chang et al. 2018 [39]

In our study, several ligands grafted on the biochip surface were evaluated, including antibodies against influenza hemagglutinin or neuraminidase. Nevertheless, the selected ligand, fetuin protein, offers the advantages including the natural interaction between influenza and host-cell receptor. Indeed, the influenza binding to sialic acid present at the surface of host-cell receptor is here used to have specific binding of HA to highly sialylated fetuin.

We demonstrated that such technology could allow for the analysis of different sample preparations ranging from purified material (trivalent commercial vaccine or virus produced on cells or embryonated eggs) up to crude material extracted from a bioproduction (viral particles released from cell-based processes). In order to allow for implementation of such tool for cell-based production processes *off-line* or *on-line* monitoring, the assay development necessitated evaluation of several parameters to ensure the pertinence of the quantification performances (ligand selection, grafting, surface regeneration etc...). Thus, sample pre-treatment with bacterial neuraminidase

was proved to be essential to avoid the degradation of the pool of sialic acids present at the sensor surface, as well as an appropriate complete regeneration of the sensor surface with optimized buffer to allow for long term analysis (> 25 samples). Following the assay optimization, we were able to provide a quantification tool presenting improved analytical performances compared to reference SRID and infectious particle quantification in-use.

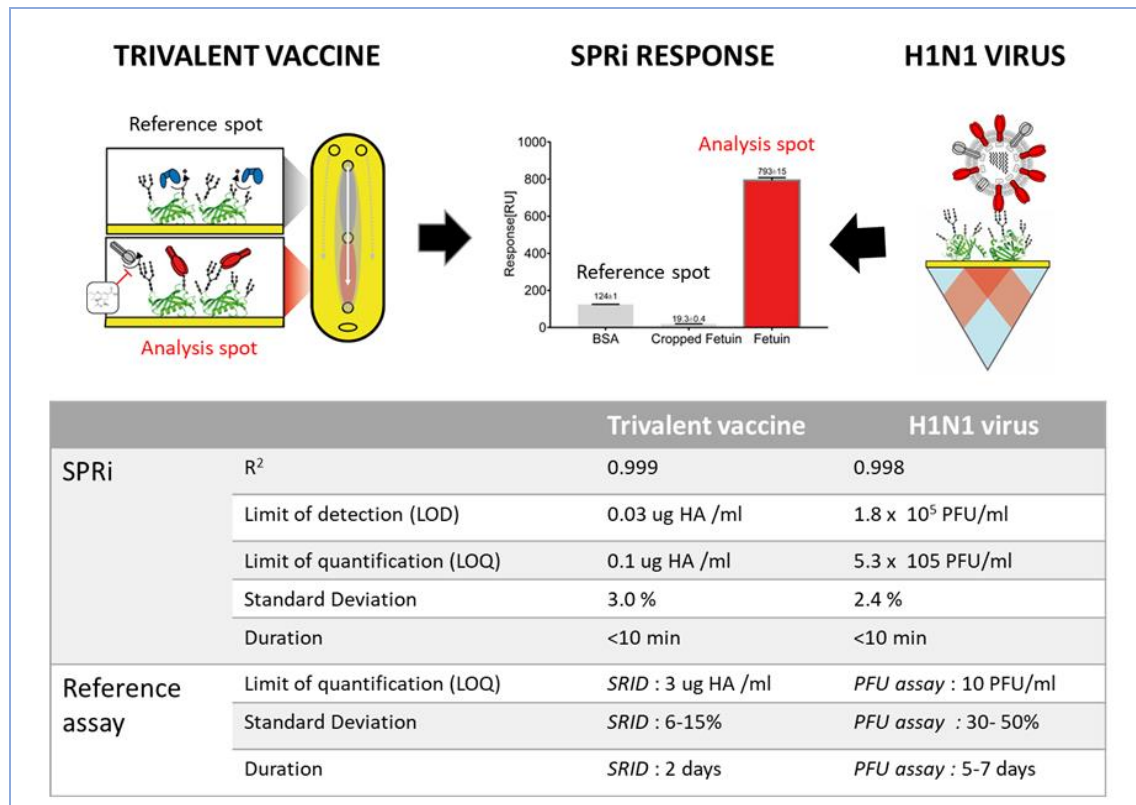


Figure 18 Analytical performances achieved with SPRi grafted with Fetuin ligand towards influenza purified antigen (trivalent vaccine) and viral particles. Adapted from Durous et al. 2019 [40].

SPR imaging technology was further implemented on-line, connected through an autosampler and a clarification acoustic filter to the cultivation vessel to allow for real-time monitoring of influenza bioactive viral particle release (see Figure 19 for system flow-chart). Such work is currently under finalization for publication.

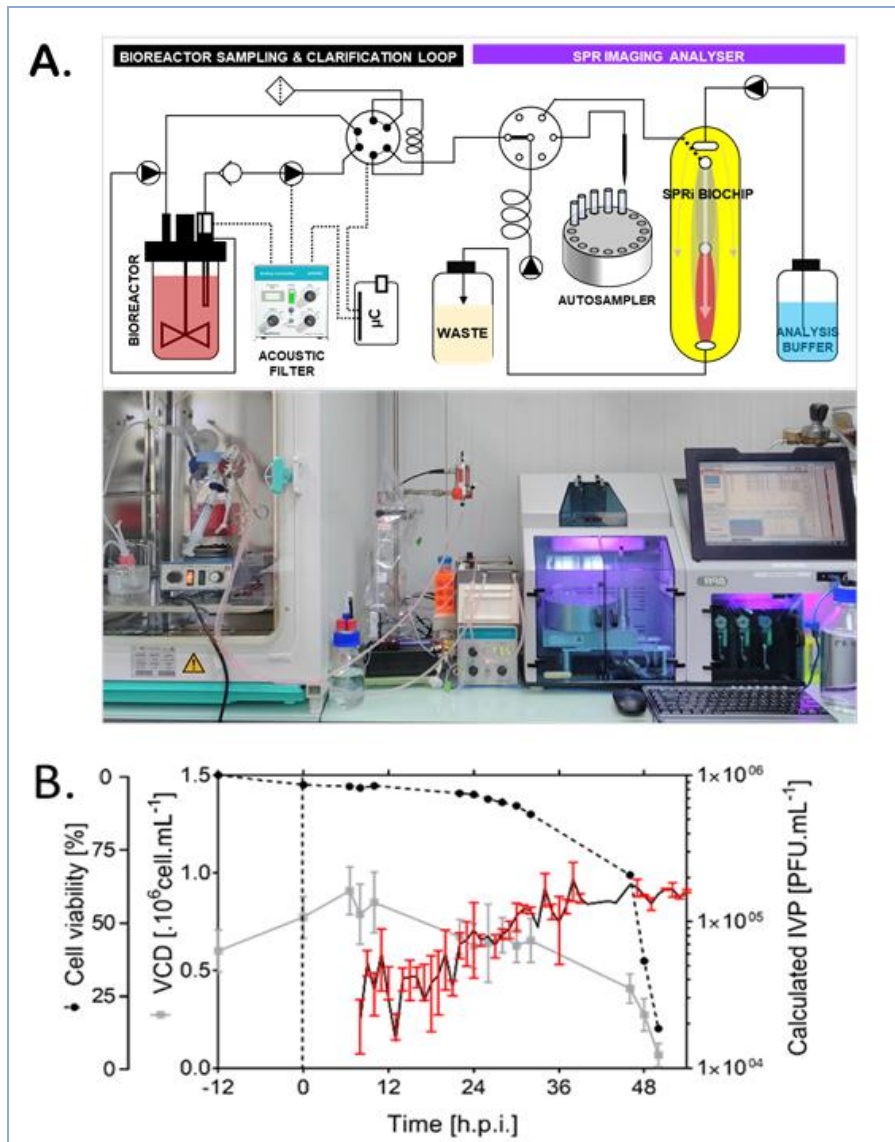


Figure 19_ A. On-line SPR imaging analyser system in-house developed to monitor in real-time viral production in cell-based culture. **B.** Influenza A /Panama/07/99 (H3N2) virus production kinetic monitored on-line. Legend : h.p.i. = hour post-infection, VCD = Viable cell density, IVP = Infectious viral particles.

Capacitance spectroscopy

To continue with capacitance probe, we explored the possible monitoring of the virus effect on cell culture thanks to their dielectric properties' changes. This strategy was applied to several virus and presented in a recent study compiling trends observed with different virus-host cell culture processes [29] (see Figure 20). More details about links between viral replication kinetics and the signal trends could be found in specific papers we published on influenza virus [20] or lentiviral productions [41].

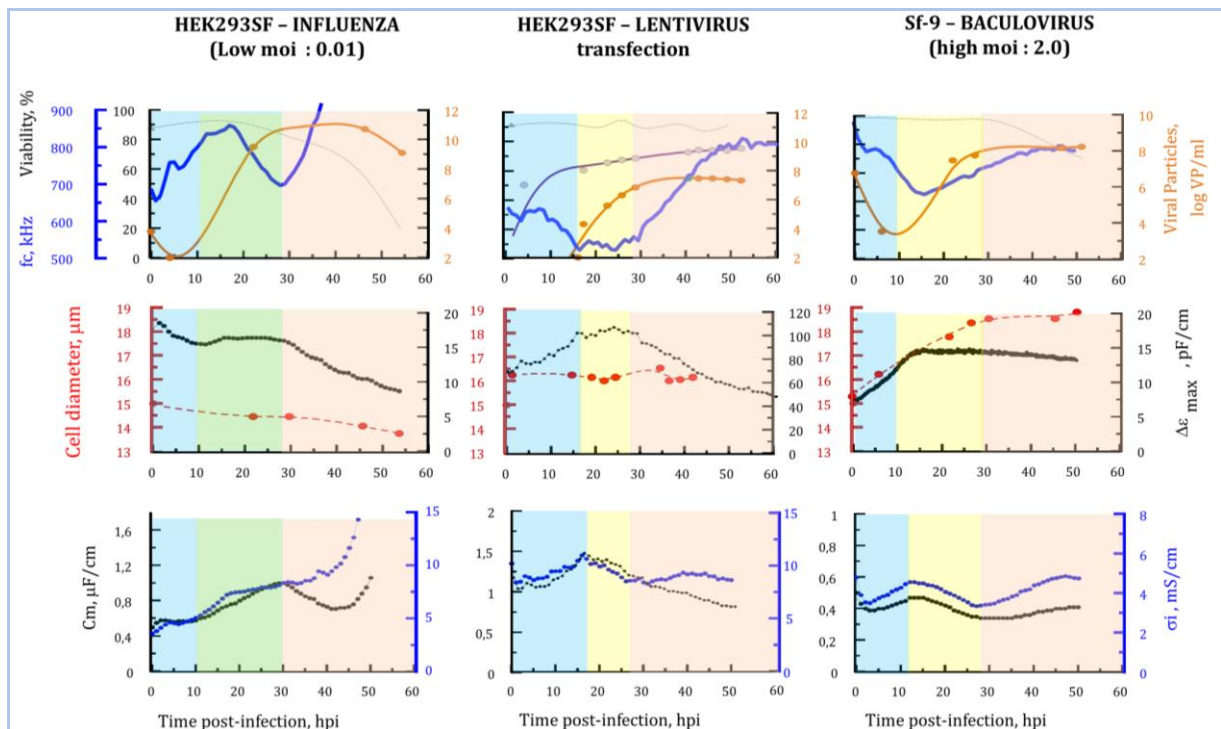


Figure 20 Viral production phases monitoring based on cell capacitance monitoring. *Blue zones: Viral entry and intracellular biosynthesis, yellow zones: Viral release, Red zones : Cell death*

To go further, we were able to build correlations between the viral production levels and the cell dielectric properties (see Figure 21). These results demonstrate that cells modifications are proportional to their productivity and the amount of virus produced. These results support the idea that cell physiological changes can be monitored and correlated with production state using cellular capacitance measurements.

Such correlations were here limited as they necessitate to tightly modulate the production levels to confirm the trends. In our case, the production modulation was performed thanks to process feeding modification and we were able to demonstrate that the control of the system was limited. To go further, a modified virus with inducible production would be a perfect tool.

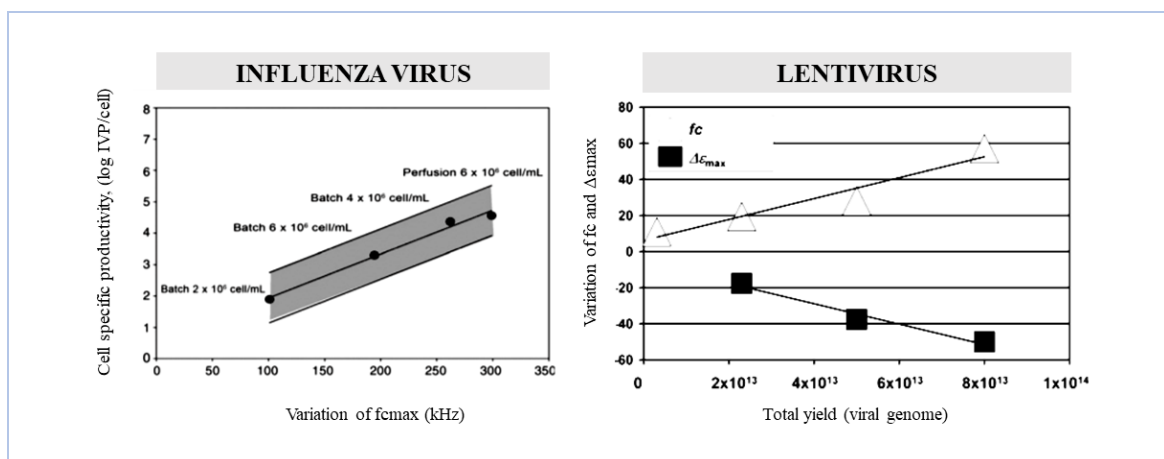


Figure 21 Viral production level monitoring from capacitance signals. Adapted from Petiot et al. [20] for influenza and from Ansgore et al. 2011 [41] for lentivirus.

Although we evaluate and developed several technologies to better understand our processes, we still lack a comprehensive method to tightly control viral production levels and quality. A lot of effort is made in this field and our last development proposing SPR technology to be implemented *on-line* is one of the tools which will eventually meet these needs.

4.3. New cell-based process development

This section is very much focused on the production of influenza virus or virus like particles although I also participated to some process development for other viruses. As an example, I realized a work for the company Oncolytics Biotech® that was evaluating and launching a reovirus as oncolytic virus. I was compiling results from different production scales from 1-l to 500-l to perform process robustness study. This work was aligned with the Quality by Design approach to understand the critical process parameters impacting end-product quality. This work was never published as it was under confidentiality agreement.

This topic corresponds to a total of 6 publications in international journals, 1 oral communication and 8 poster communications in international conferences. It also led to a international that I initiated and coordinated between 2014-2016. This program was built between Virpath laboratory I was belonging, A.Star Singapore institute and McGill university and funded by a European Grant Marie Curie IIF FP7 Fellowship. These researches were also the aim of several other collaboration I started with for example the laboratory of CNRS UPS 3044 of Ales on Baculovirus and Therapy. Within this topic I supervised of 1 Canadian Master student (2 years program), 1 French Master student and 1 pharmacist-engineer student.

4.3.1. Novel influenza processes for viral seed stocks or vaccine production

The influenza vaccine field facing recurrent pandemic coming from zoonotic virus is classically under pressure to speed up the production timelines and provide easy to produce antigen lots. Now a lot of new strategies propose alternatives to the standard embryonated egg strategy at different steps of the product manufacturing [42–44]. It is now largely recognized that cell culture-based processes allow for greater capacity, process control and more reliable and well-characterized products.

First, the cell-based production processes were evaluated with the standard infection process approach. Thus, both mammalian [1,22] and avian [3] cell platforms were evaluated, demonstrating that production efficiency, timelines, production levels are a lot dependent upon the cell physiology, the process implemented and of cell production platform. As demonstrated in the following compiled graph (Figure 22), for similar process protocols, we could reach highly variable viral titer levels as well as production kinetics with strains collected on human, avian or swine host. This is linked to the capacity of influenza strains to bind specifically to sialic acid presenting α -2,3 or α -2,6 glycosidic bonds at the surface of cell receptor. As such glycosylation pattern is highly specific or of the host or of the cell localization (low or high respiratory track for human), we understand why the choice of the production cell line can strongly impact viral replication.

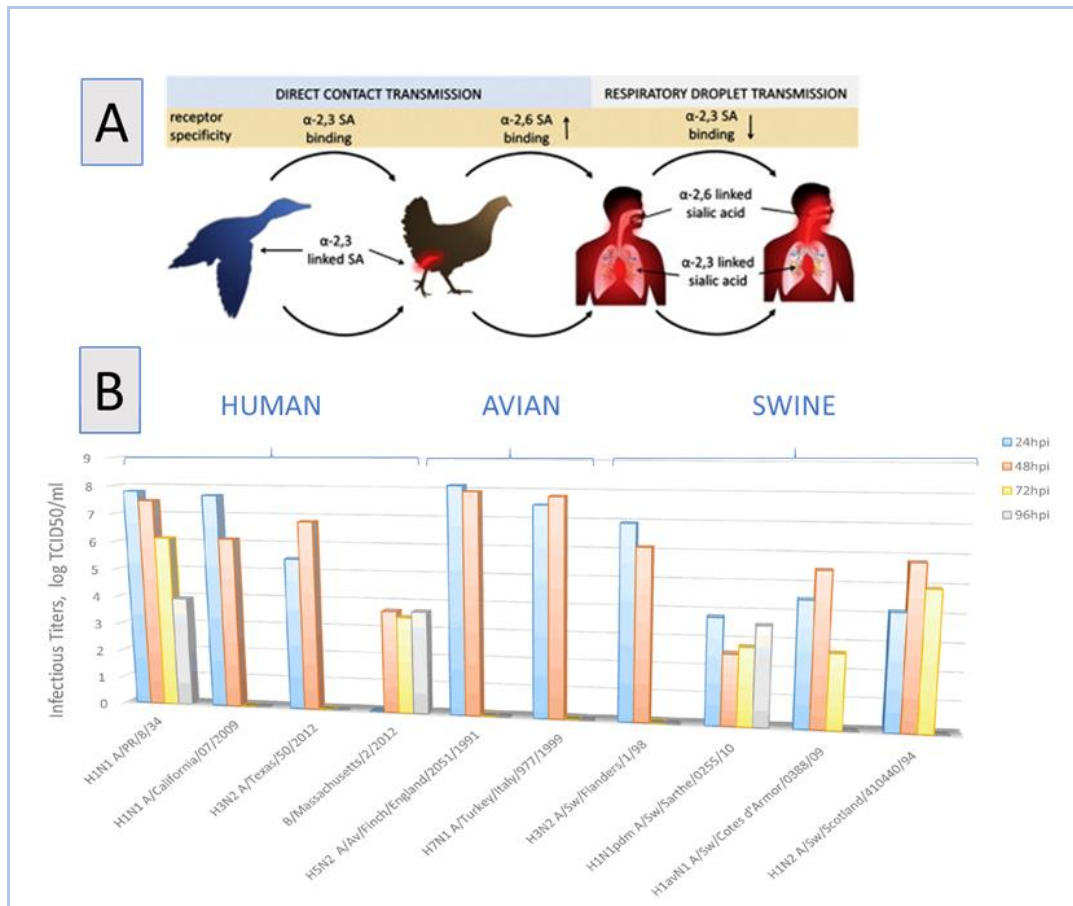


Figure 22_A- Host or tissue specific repartition of cell receptors. **B** - Viral strain production from different hosts on avian DuckCell™-T17 cell line. Adapted from Mair et al. and Petiot et al. [21,45]

However, in spite of the recent licensing of cell-culture manufactured influenza vaccines, responsiveness to an influenza pandemic threat stays strongly attached to the necessary time to reach the “master viral seedstock” or Candidate Vaccine Virus (CVV), as reported by WHO Informal Consultation report of 2015 entitled “Influenza Vaccine Response during the Start of a Pandemic” [46].

To answer this needs, a reverse-genetic approach applied directly in suspension at large scale was evaluated [47]. Indeed, the target was to provide a rapid and efficient production of influenza virus to faster provide viral seed stock to manufacturers. This was enable as it was based on two preliminary proofs-of-concept; first the HEK293 cell large-scale transfection [48] and second HEK293 influenza replication and production at large-scale [1,22]. Such work demonstrated for the first time that a suspension cell line can be efficiently transfected at bioreactor scale with eight reverse genetically engineered plasmids. The further infection amplification within the bioreactor allowed to produce high titers of influenza PR8 strain in two steps (transfection/rescue and infection/production) with virus yield reaching 10^7 IVP/mL [47]. This approach could deliver a CVV for influenza vaccine manufacturing within two-weeks, starting from HA and NA pandemic sequences, indicating that HEK-293 cell culture is a robust system to obtain viral seed stock for influenza production within a very short period (9 days).

4.3.2. Virus like particles as influenza vaccine candidate or protein carrier for vaccine development

In recent years, we were also focused other cell-based alternatives. Medicago plant-based strategies and Protein Science insect cell subunit vaccine solutions are successful examples of the recent progress performed to develop of recombinant vaccines and provide rapid solutions to produce vaccine lots. The advantages of such solutions are obvious as they allow for rapid production of any influenza strain, or strains combination by cell transformation (either transfection or transduction from a viral vector). It is also a great alternative to produce viral structural forms (either rosette for insect cells, or full virus like particles VLP for plant cells) that are closer to the native influenza virus morphology to better enhance immune B- and T-cell responses. Nevertheless, as for any new cell-based production strategy, production methods and product characterization allow to identify bottlenecks and limitations of the strategy.

We were for many years evaluating this technology and the interest of different cellular platform to reach maximal cell productivity of flu-VLPs [49,50]. Developing and comparing different processes allowed us to identify strong bottlenecks in the viral particle release for influenza VLP production.

As an example, comparing production in Sf-9 insect cells and in HEK-293 human cells demonstrated that structural protein matrix M1 was not correctly addressed and packaged in the VLP in the case of HEK-293 cells (Figure 23). This led to a 10 times lower production efficiency in this cell platform. In other teams, such limitation of the particle release was overcome by creating chimeric VLP including Gag-HIV matrix protein to replace native influenza M protein [51].

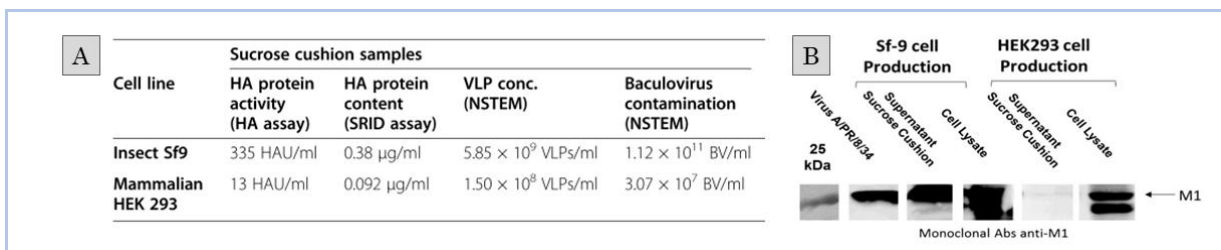


Figure 23 Comparison of influenza Virus like particle (VLP) production lots from HEK293 and SF9 cells. A- Comparative quantification of VLP lots. B- Western Blot for anti-M1 influenza protein for the two cell platforms. Adapted from Thompson et al. 2015 [50].

The contamination of the produced flu-VLP was also risen in these studies as the main cell platform in use, Sf-9 cells, exhibit a strong co-production of viral vector (baculovirus) at the same time as the VLP productions. As detailed in our recent review of the topic, multiple purification strategies can be used to allow for separation of these contaminants [52]. Developing such recombinant production strategies with high level of cell-based contaminants and such low levels of antigens productions forced the scientific community to propose improved purification strategies, never reached in the past for embryonated egg productions.

Virus like particle (VLP) forms were early identified as interesting carriers for antigens from other pathogen origins. Thus, several chimeric VLPs based on HIV, Hepatitis B or influenza virus were proposed in the last fifteen years. Similar approach was chosen to carry Malaria antigens and elicit T-cell response which are strongly impacting the quality and lasting of the protection against Malaria. In that case, four target malaria epitopes were chosen to be integrated in HA protein.

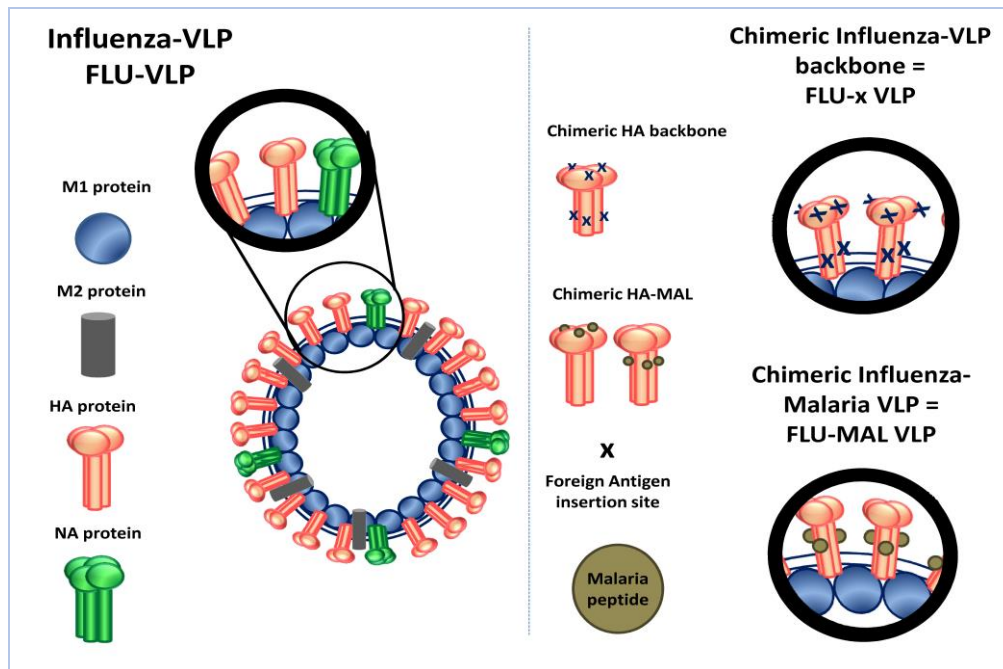


Figure 24_ Chimeric influenza VLPs to carry foreign antigens. Here targeted foreign antigens are epitopes of MAEBL or CS malaria proteins identified as interesting vaccination epitopes for immunisation against *Plasmodium yoeli*.

Two chimeric production strategies were chosen to obtain influenza-VLPs in sufficient quantities to immunize mouse models, (i) the transfection of HEK-293 cells with plasmid carrying influenza genes and (ii) the Sf-9 cells with baculovirus carrying influenza genes. The target was to achieve 1mg of chimeric VLP purified at 80%. A concomitant development of both production process was conducted to select the best production platform for these chimeric VLPs. Both platforms presented some challenges and limitations impeding the sufficient production influenza VLPs.

Production levels was the first critical step to overcome. HEK293 cell platform necessitated a long fine-tuning of influenza protein ratios to achieve correctly formed virus-like particles thanks to transfection. In that case, and as identified in previous study, particle correct assembly and their later budding was the limitation. Therefore, the ratios or origin of matrix proteins (M1/M2 or Gag-HIV) was under study in our assays (see Table 3).

Table 3_ Influenza VLPs produced by transfection in HEK293 cells. Variation of proteins ratios, VLP particles counted by TRPS and presence of exosomes on EM grids.

		VLP-A	VLP-B	VLP-C	VLP-D
Proteins for VLP construction	HA H3N2	1µg	1µg	1µg	1µg
	NA H3N2	0.75µg	0.75µg	0.75µg	0.75µg
	M1 H3N2	0.75µg	1 µg	1 µg + 1 µg	-
	M2 H3N2	0.25µg	0.25µg	0.25µg	-
	Gag-HIV	-	-	-	1 µg
VLP particles counted		6,72E+10	1,30E+10	1,36E+10	2,06E+10
Presence of vesicles		+	+	+	+

Then, the presence of cellular contaminants was the second main target of this study. Indeed, the secretion of cell exosomes presenting similar sizes and morphologies as the VLP produced necessitated to develop dedicated purification protocols and quantification tools. As for the baculovirus/insect cells platforms, similarly to previous work, we were not able to separate VLPs from contaminant baculoviruses (see Figure 25). Consequently, within the timeframe of the funded program, the malaria epitope integration was never achieved although integration sites in the hemagglutinin were selected.

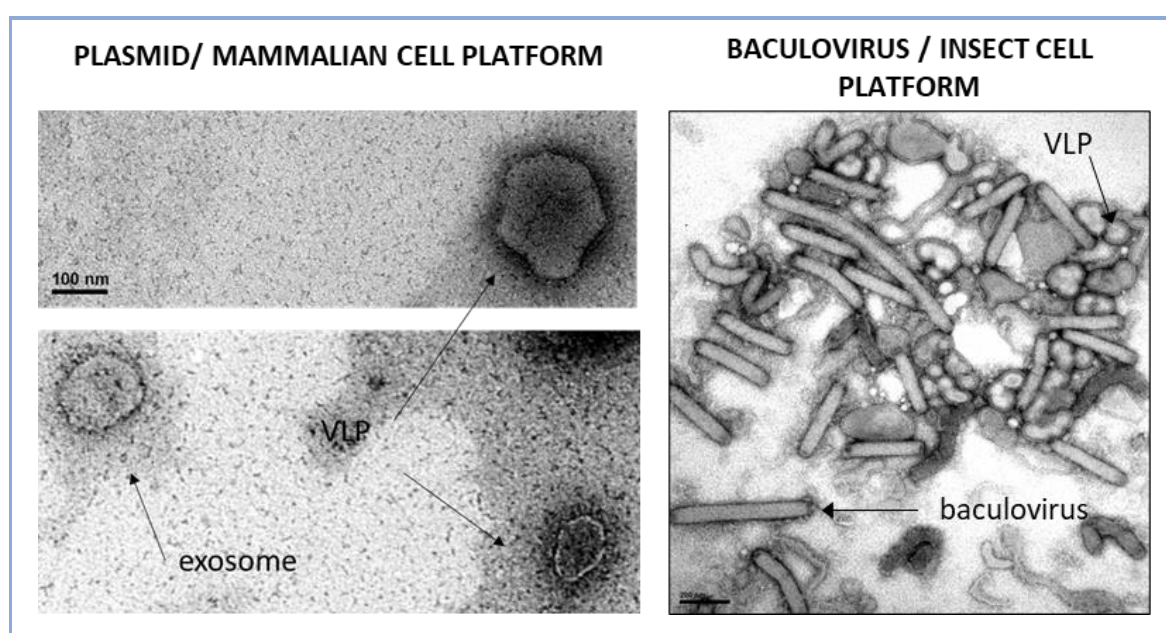


Figure 25_ Comparative analysis of purified influenza-VLP by electron microscopy.

4.4. Cell-based process development for regenerative medicine

In 2017, I joined the ICBMS-3d.FAB platform to provide expertise on cell culture and bioprocess development for tissue production applied to regenerative medicine. As detailed in the introduction section, these research activities rely on a transfer of the knowledge, know-how and methodologies I developed in the past ten years for virus and viral vector cell-based productions. It consists in the continuity but also the extension of my research activities to a broader cell-based production field (regenerative medicine & cell-based

therapies). As an example, the following part will detail a central project of these activities and complementary information will be further developed in the section describing the research program planned for the next 5 to 10 years.

This topic allowed to recently publish 1 publication in international journals and to present 1 poster communication in international conferences.

4.4.1. Bioprocess maturation of 3D printed dermis

3D printed tissues are interesting tools for both regenerative medicine and *in-vitro* models in alternative to animal testing. For 5 years, 3d.FAB platform developed bioprinting processes, bioink and maturation protocols to generate living tissues from primary cell culture (see Figure 26). Several publications from the team now established the proof of concept of these processes for diverse tissues including skin, cartilage, retina etc...[53,54]

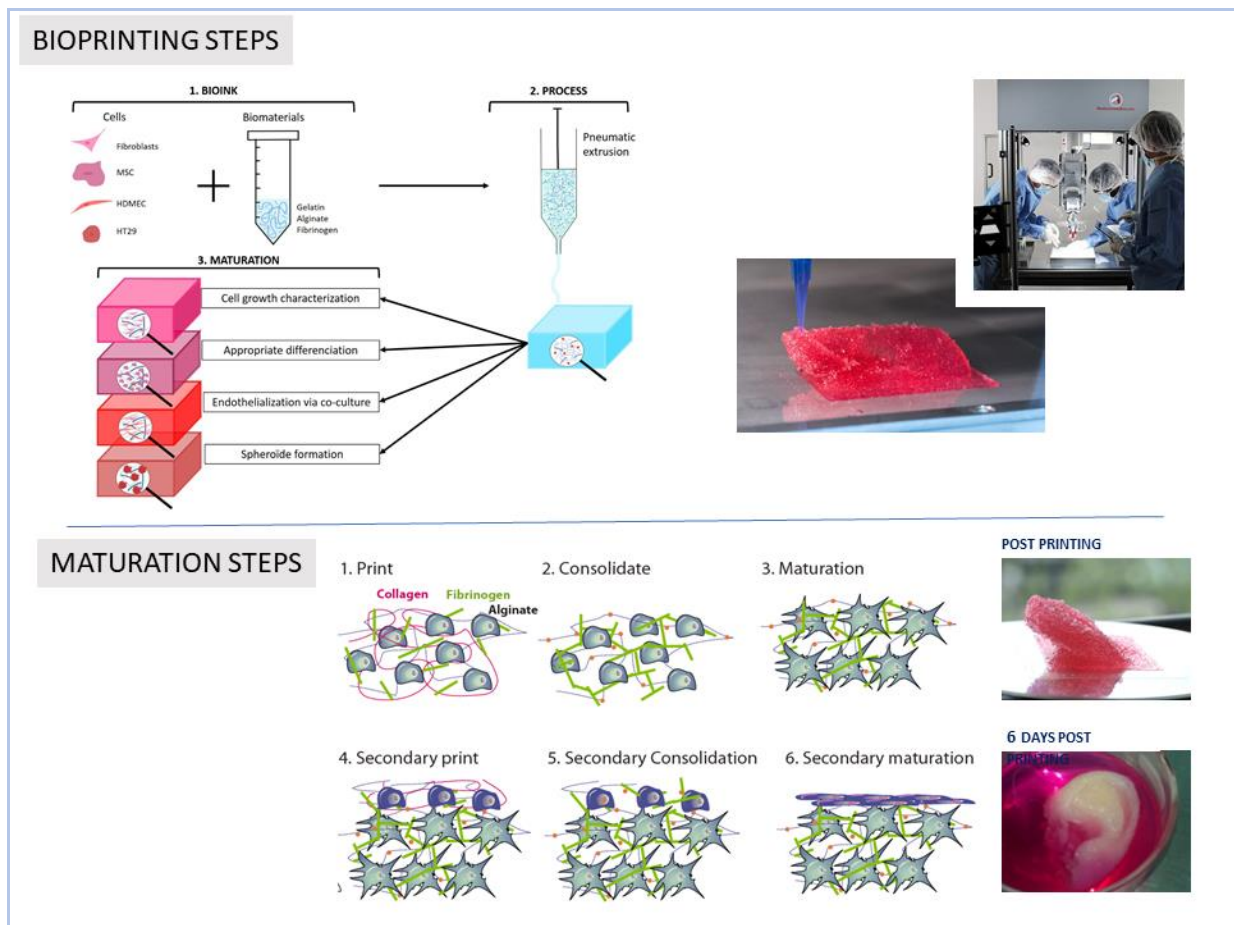


Figure 26_ Bioprinting of live tissue. Bio ink preparation, printing, consolidation and maturation process.

To be anatomically and physiologically relevant, such 3D tissues shall have a minimum size, above one cm^3 and exhibit some mandatory features as for instance, compartmentalization or barrier/diffusion functions, transmission of the inflammatory response of innate immunity or cellular paracrine/endocrine communication. Vascularization and micro-vascularization networks as well as controlled heterogeneity were soon identified as the limiting step to recapitulate physiological behavior of the

produced tissues. These vascular structures are indeed mandatory, first to ensure tissue growth and viability, but also to recapitulate fully the *in-vivo* tissue complexity, functions, and interaction with external environment.

One of the strategies pursued by the team to recapitulate such functions is to induce tissue maturation thanks to bioprocess-controlled culture conditions. One of the major targets is to master the flow path and its effect on a 3D predefined tissue structure and is entitled “dynamic maturation”.

Dynamic maturation shall lead to:

- Locally determined shear stress, beneficial to induce capillaries and vessel structures self-organization.
- Controlled supply of nutriment and active molecules to the tissue embedded cells enabling 3D heterogeneous tissue maturation.
- Controlled seeding of the 3D tissues with specific cells through flow trajectories. This strategy will enable the dynamic localization of endothelial vascular cells at discrete locations.

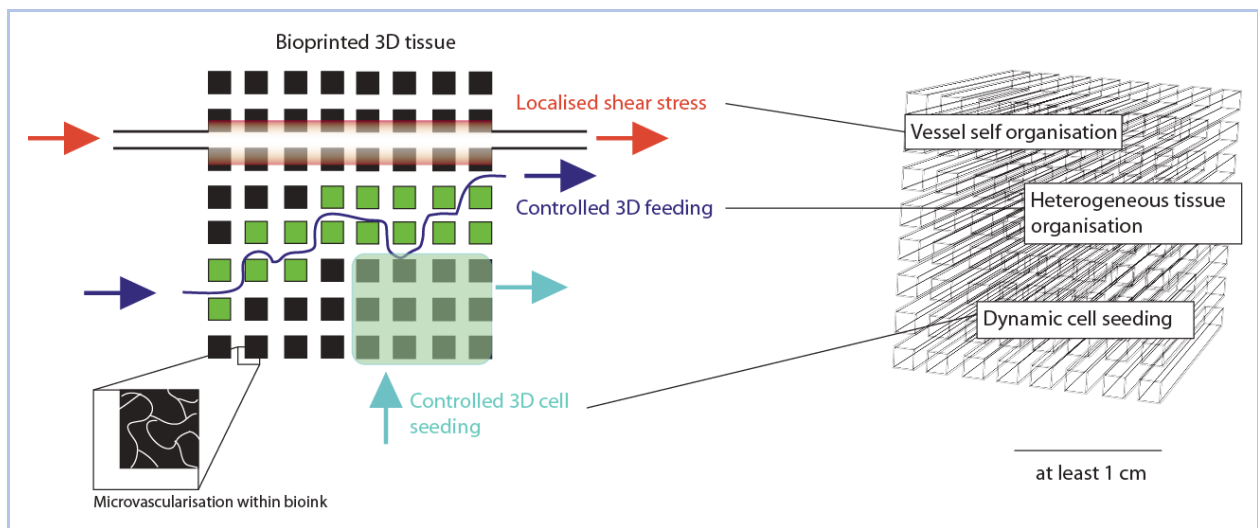


Figure 27_Dynamic maturation impact on 3D printed pre-defined structure of tissue.

This strategy implies, as for previous cell-based process development, a high knowledge of the cell growth/death kinetics and mechanisms (response to shear stress and mechanical stimulation), an enlightened knowledge of the cell metabolism and physiology (specific feeding, growth factors) and a good description of the maturation phases and phenotypic descriptions of matured tissues (extracellular matrix secretion, presence of specific differentiation markers). As for other cell-based process development, both simulation / predictive tools and specific analytics must be developed. This will be further developed in the section 5 describing my research project.

As preliminary results, this research program was initiated in collaboration with the laboratory LRGP from Nancy on a 3D printed cube of conjunctive tissue of 8 cm³ (Figure 28). The goal was to evaluate impact of flow-path, simulated by Computational Fluid

Dynamics (CFD) on the tissue vascularization and maturation process and to compare with standard static cultivation in CO₂ incubators.

This work allowed to identify that the developed procedure enables, in one hand the conservation of the internal porosity of the bioprinted tissue, leading to a constant and homogeneous perfusion of the entire tissue, and in the other hand the development of a clear organization in connected endothelial cell networks. The lower production of collagen, essential component of the extracellular matrix, within static cultivation conditions clearly indicates a lower maturation rate of the tissue. Similarly, the human dermal microvascular endothelial cells' organization demonstrates a clear difference between dynamic and static culture conditions indicating the impact of flow on the maturation and cellular organization process. Based on these observations, research program is now under realization within the team to link cell cultivation process and context with the tissue fate (maturation rate, functionalization).

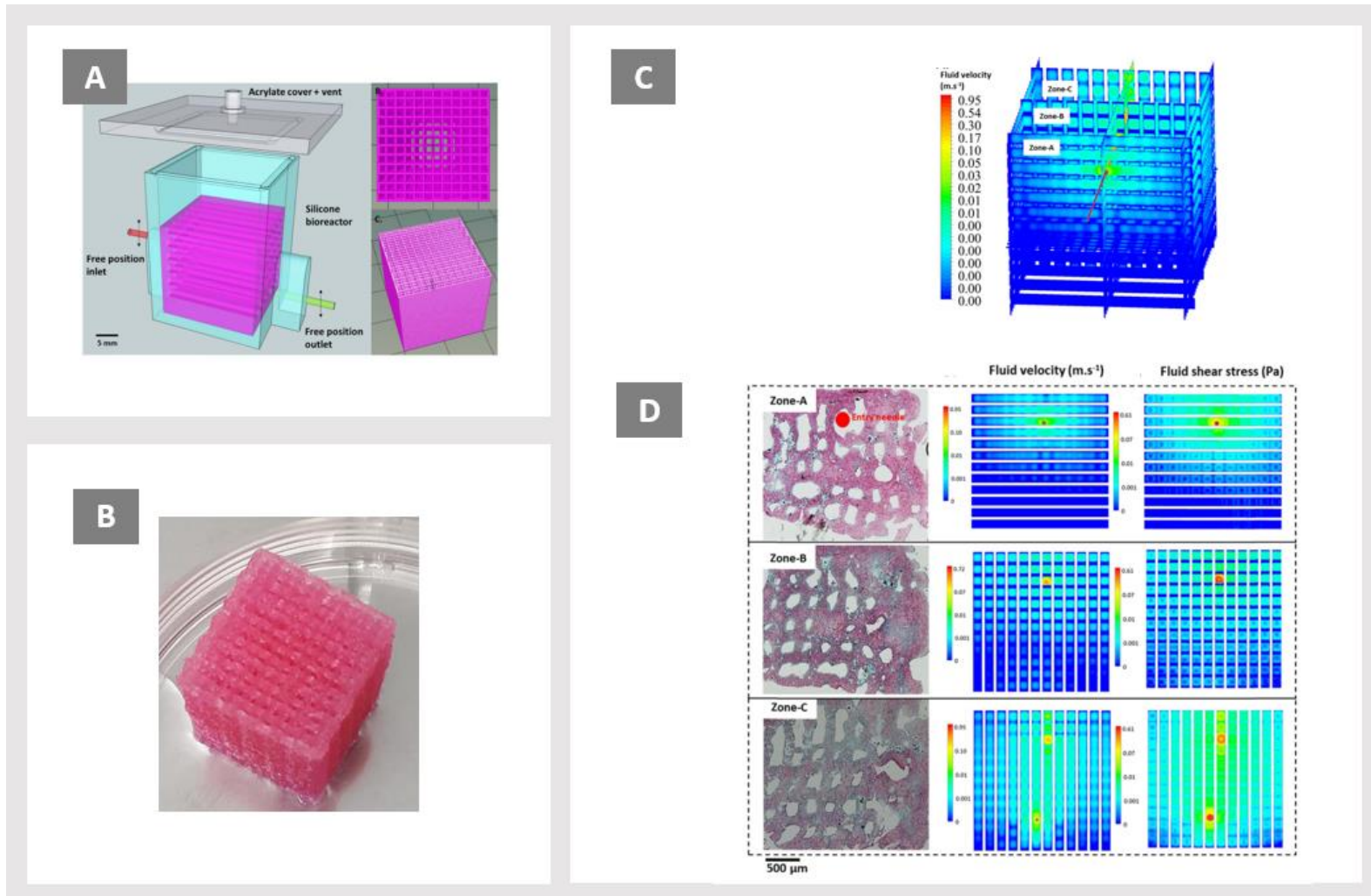


Figure 28_ 3D printed perfusion system for dynamic maturation of vascularized dermis. A: View of 3D printer bioreactor with its inlet and outlet free positioning (through needle puncture) and 3D view of the bioprinted large tissue showing internal macro-porosity. B: Bioprinted cubic 8cm³ dermis tissue. C: Fluid flow velocity fields from CFD simulation of three different zones within the large bioprinted tissue. D: Characterization of 500 μm sections of the bioprinted tissue after 12 days of dynamic culture. Full-size view of histological sections of bioprinted tissue (masson trichrome: green color represent collagen) and corresponding fluid flow velocity fields and fluid shear stress fields from CFD simulations. Adapted from Pourchet et al. 2019 [55]

5. Project: Bioprinted tissue bioprocesses

My current research project consists in improving the comprehension and mastery of biological phenomenon involved in bioprinted tissue maturation and their physiological functionalization thanks to bioprocesses. This approach integrates (i) the evaluation of feeding strategies (addition of growth or maturation factors, nutrient feeding), (ii) the development of dedicated analytics, (iii) the development of specific bioreactor vessels and cultivation strategies (see Figure 29).

This research program is now gathering several research collaborations with academic teams or platforms (LRGP : CFD simulation; CERMEP : MRI; Histology : Primatiss LBTI platform) and companies (Sartorius : Bioreactor and on-line analytics; Kaiser Endress : Raman; Aber : Capacitance; Ipratech : Bioreactor set-up)

One main funded contract signed with Sartorius Company in december 2019 is supporting these activities. This research collaboration aims to develop bioprocess applied to tissue engineering and tissue bioprinting. Within this program I will coordinate the work of at least two persons, one PhD student (Laura Chastagnier) for which I am presenting this HDR and one post-doctoral fellow funded by Sartorius and planned to start in 2021.

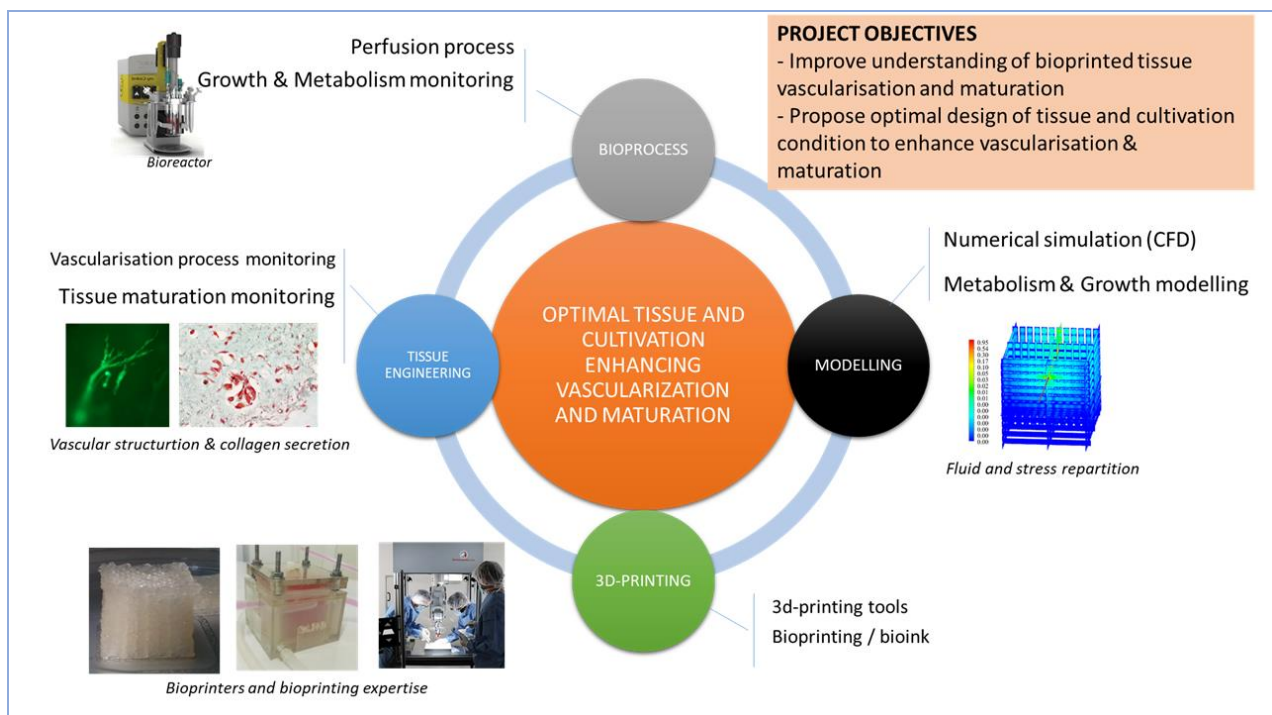


Figure 29_ Bioprocess applied to bioprinted tissue cultivation and production

5.1. Feeding and cultivation strategies

As demonstrated in our preliminary study [55], cell feeding rate are of strong importance to reach matured and functional tissue. As for standard cell-based process, deep evaluation of cellular nutrient needs is necessary at this stage of development. Recent work was directed towards the evaluation of the cellular specific needs in oxygen and glucose while growing and maturing in the tissue. Evaluation of the nutrient diffusion rate within the bio-ink and a matured dermis was also initiated (Figure 30). Such metabolic description is planned to be deepened with both standard quantification assays (enzymatic quantification on culture supernatant) and more advanced strategy (Raman spectroscopy). Next step is to provide, thanks to fed batch or perfusion strategy, the exact amount of nutrient to get closer to natural environment met in organisms or organs. This part is linked with the development of dedicated bioreactors set-up for tissue engineering (see section 5.2).

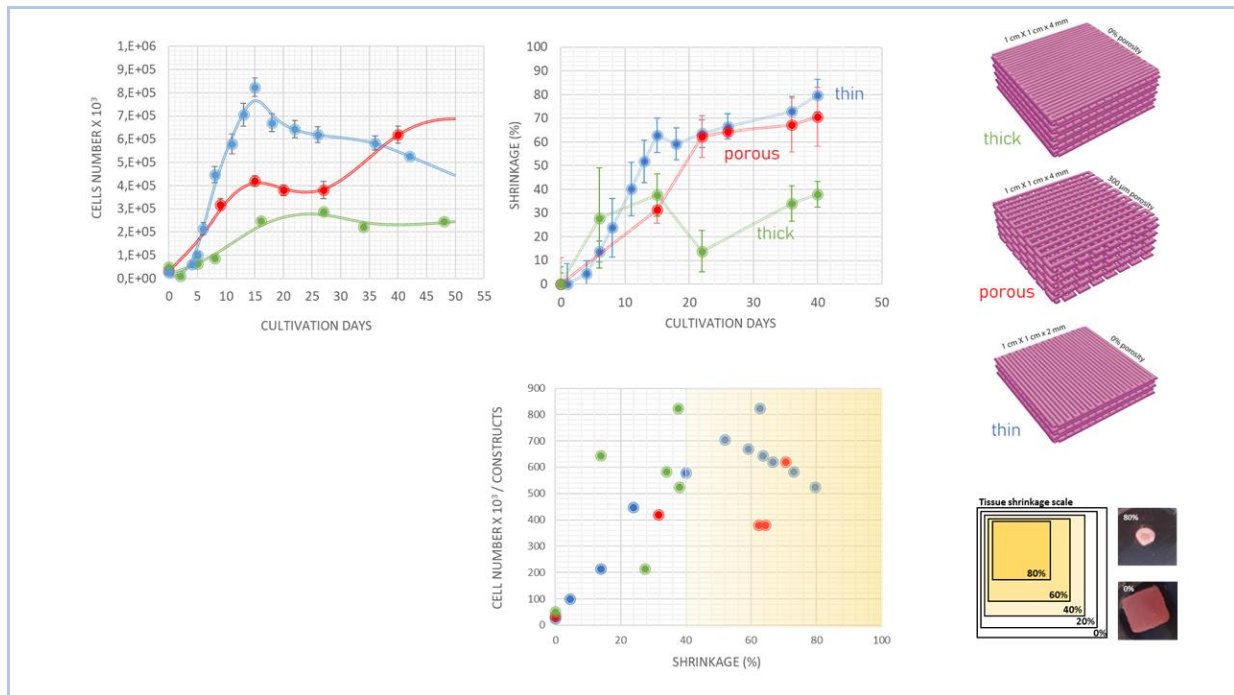


Figure 30_Evaluation of growth and tissue shrinkage for different bioprint geometries, thick (10 x 10 x 4mm), porous (10 x 10 x 4mm with 300um porosity) and thin (10 x 10 x 2mm). Extracted for manuscript in preparation Chastagnier et al. 2020.

Simultaneously, screening of specific growth factors or protease inhibitors are ongoing. Indeed, tissue maturation steps are multiple and commonly induced by secretion/induction of specific biological molecules (EGF, FGF, TGFb, ...). On the other hand, extracellular matrix (ECM) secretion is in our case controlled by the cellular mechanisms of environment remodelization. This process consists in cellular secretion of Metalloprotease enzymes to degrade the ECM and collagen fibers before secreting neo-synthesized cellular collagen. These biological processes explain why the maturation duration is over 20 days of culture.

Thanks to feeding and/or addition of specific molecules, we target to master such biological processes and to rationality drive the maturation duration while reducing it if possible, below 10 to 15 days.

5.2. Bioreactor design

The aim of this part of my research is to propose cultivation systems allowing to tightly control the cellular and tissue environment. Here “controlled environment” is important to be described as the standard bioreactor proposed for tissue cultivation are commonly placed in incubator with no regulation of pH and oxygen. In our case, we want to reach dedicated system allowing, as for cell-based industrial production processes, to measure and retro control at least the 3 essential parameters for mastering cell cultivation, i.e. temperature, pH and oxygen supply to the cells. To reach such target, the maturation of bioprinted tissues, originally constituted of very few cells, which then proliferate and reconstitute their environment to have functional tissue properties, involved several constraints.

First, the low number of cells present in the construct at the beginning of the tissue development necessitate and adjustment of the cultivation vessels volumes. As already know in cell-based processes for years, cell growth is commonly limited by the dilution of the cell’s autocrine growth factors and mediators in the cultivation media leading to the so called “latency” cultivation phases [56]. Second, the specific shape of each tissue necessitates dedicated cultivation vessel design. Third, tissue maturation and cell specialization can be induced by mechanical stimulation (flow for endothelial cells organization; compression/shear for osteoblasts...). Fourth, the monitoring tools commonly adapted to incubator cultivation or to large-scale cell culture are not satisfactory to analyze large bioprints cultivation.

This is why, our strategy aims to resolve these three points by (see Figure 31) :

- **Developing our in-house small volume (max 150ml) cultivation system dedicated to tissue maturation.** This is part of a new collaboration with the company Sartorius positioned as an equipment provider for pharmaceutical companies in the bioprocess field.
- **Dedicated simulation tools to prepare the design of the cultivation vessels.** Here the CFD is used to describe the flow path, velocity and shear rate within the tissues. It is also used to simulate the nutrient feeding rate of each tissue sections. A combined study of tissue and cultivation vessel shape allows for instance to propose geometry allowing for homogeneous or heterogeneous cultivation conditions.
- **Design and print cultivation vessels dedicated to the maturation of specific tissues.** This allows for shape and size adaptation and for multiple flow path within the tissues. Thus, planar vessels will be proposed for skin tissue, tubular two-flow vessels for vascular tissue and cubic vessels for conjunctive tissue to study vascularization. It also allows for integration of specific mechanical stimulation equipment (see section 5.3).

- **Dedicated analytics implemented on both the regulation and the cultivation vessels.** This will be further developed in a specific section.

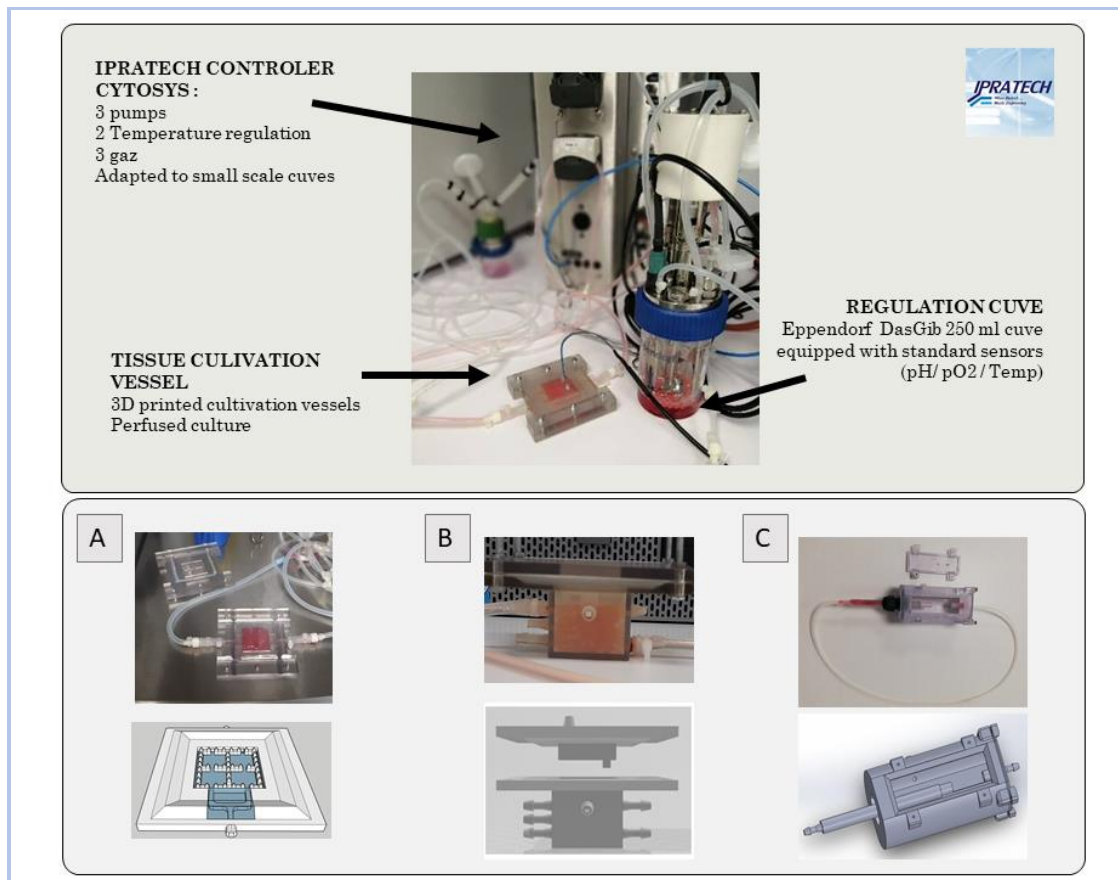


Figure 31_ Tissue cultivation bioprocess systems in-house developed. **A. Planar skin tissue bioreactor, B. Cubic vascularized conjunctive tissue bioreactor, C. Tubular vascular tissue bioreactor**

5.3. Mechanical stress impact on tissue and cell fate

It is known for years that cells, ranging from stem cells up to highly differentiated cells, are impacted by mechanical stress [57,58]. Bioprinting process principle is to extrude embedded cells in a biomaterial of known viscosity through needles or tips. Such process is commonly inducing different ranges of stress (shear stress) accounting for cell degradation, cell death or cell physiological changes. We have in-house developed a simulation tool enabling to predict the ranges and intensities of shear stress applied to the cells while they are printed in specific conditions (see Figure 32).

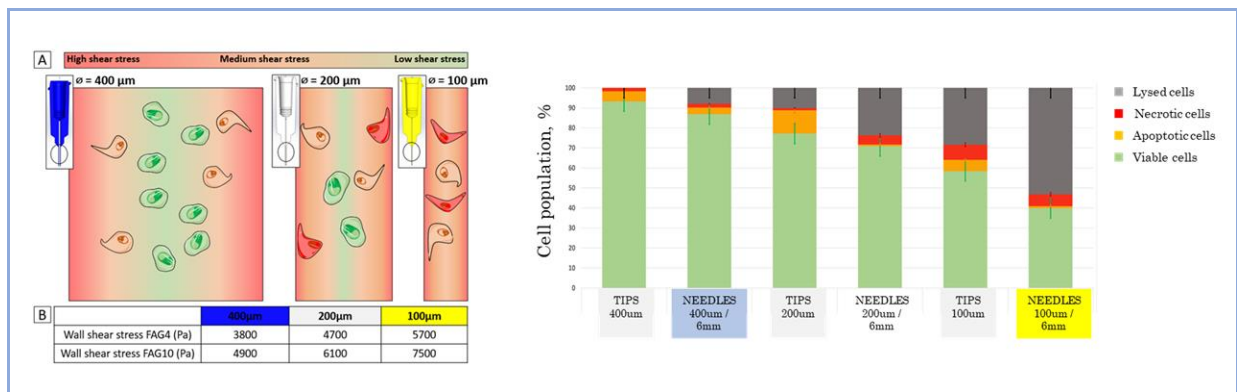


Figure 32_Simulation and effect of shear stress on cells while printing. A & B. Simulation of wall shear stress with two bioinks presenting gradual viscosity (FAG10 > FAG4) and different printing nozzles (tips or needles of different size). B. Effect of shear stress on cell population after printing with different nozzles and using FAG10. Adapted from Lemarié et al. 2020 (paper in preparation).

To go beyond this study, a project entitled CellStress aims at describing the cells ability to be printed and their physiological fate, thanks to a microfluidic tool applying precise shear stress. This project will allow to characterize the cell samples (either primary cells extracted from patient or standard cell lines) to predict their ability to be printed and to recover a fully functional tissue.

Mechanical stimulation and stress are also envisioned as positive actuator of cell differentiation and of tissue maturation. Research programs are currently starting in the team to (i) master the level of shear stress induced by the nutrient flow thanks to CFD simulation, (ii) master the sequence, strength or orientation of shear and compression thanks to dedicated equipment. Such programs are fully integrated in the bioprocess development approach. A Master student is currently developing a cultivation vessel to adapt mechanical controlled stimulation within a perfused and regulated tissue cultivation vessel.

5.4. Dedicated analytics: off-line and on-line tools

Our strategy to better characterize and understand the bioprinted tissue maturation is strongly connected to the analytics. Indeed, we aim to reach a better characterization of our biological systems, identifying the growth/maturation phases of the tissues, with the use or development of off-line & destructive samples techniques, and then to propose tools which would allow for tissue growth and maturation monitoring on-line avoiding sample loss (Figure 33).

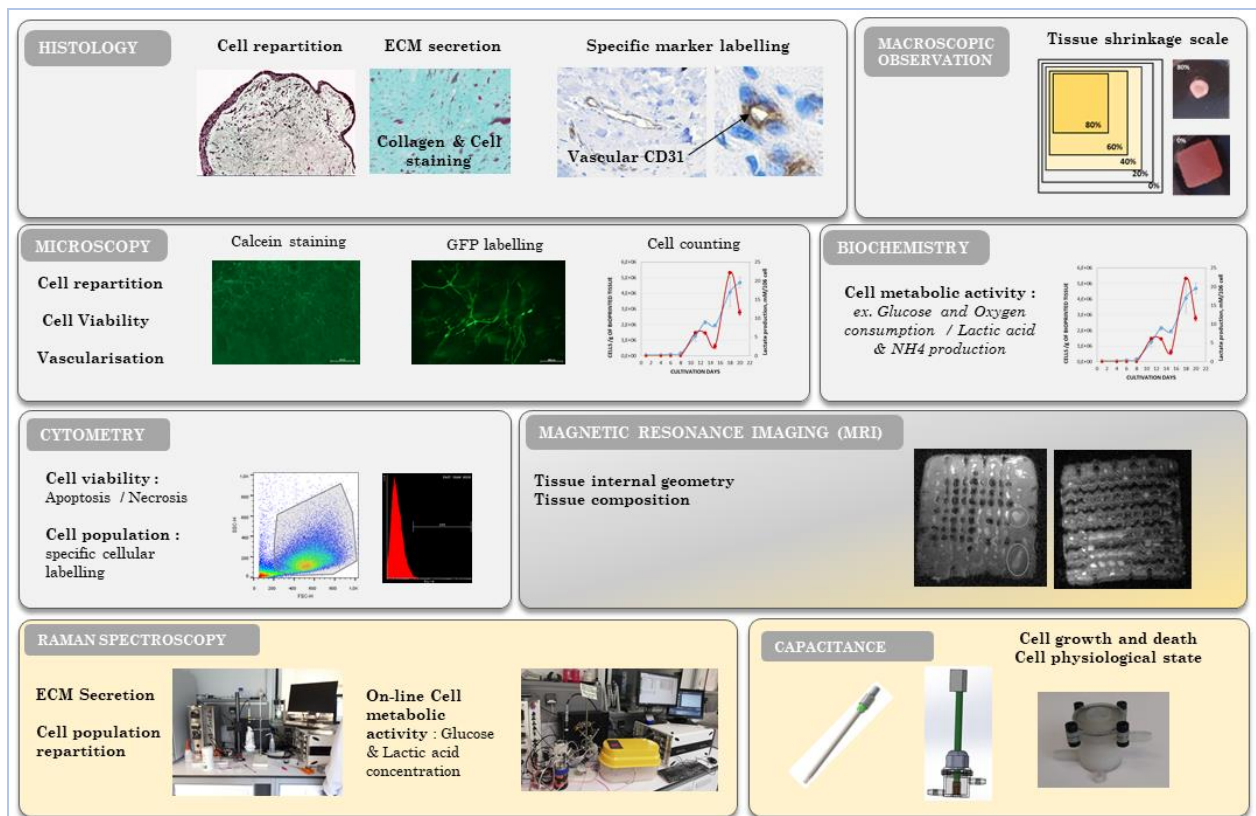


Figure 33_ Analytical tools implemented for tissue quality characterization. Grey: Off-line destructive analyses, Yellow : On-line analyses.

Until now, standard qualification of tissue quality and maturation were performed thanks to destructive assays. In the tissue engineering field, reference characterization methods rely on histological cuts of tissues embedded in paraffin with a further coloration or labelling of the biological targets (Masson trichrome, CD31/ EN4 : vascularization, ...). Although such assays allow for description of cell repartition and tissue organization and functionalization, it is too qualitative to be integrated in a bioprocess approach. Thus, complementary protocols with off-line assays were and are still experimented to propose better characterization of the tissue maturation timelines.

Thus, macroscopic observation, cell counting after tissue dissociation, quantification of metabolites released in the culture supernatant, are investigated to provide such information. Cytometry protocols were also developed to be implementable on bioprinted tissue pre- and post-maturation to be able to reach more advanced targets. Indeed, part of the team research is now focused in the printing of stem cells or iPS cells to induce cell differentiation within the bioprinted constructs.

On the top of such *off-line* quantification and characterization strategy, the goal would be to have analytical tools implemented close or on the tissue cultivation vessels in order to monitor in real-time the tissue evolution. This aims at providing a fully integrated bioprocess to produce tissue for regenerative medicine and implantation.

Tools currently evaluated are either coming from my previous expertise in cell-based process or are tools generally used for *in-vivo* analyses. Our main constraint here is to be

able to acquire data inside large and dense objects. As examples, here are the approaches that were already tested or envisioned:

4.1.1 Raman spectroscopy

Raman spectroscopy could either be applied to acquire spectra of culture supernatant circulating within the tissue thus allowing for monitoring of its secretion and consumption, as already applied in other cell-based processes, or directly on the tissue surface to acquire spectra of the biomaterial evolving with cells secretion and ECM remodeling. Both strategies are currently explored. The first one will allow the monitoring of main central carbon metabolites (glucose, lactic acid) which are directly correlated to cell growth (as demonstrated in our current studies: Chastagnier et al. manuscript in preparation). This will allow for *on-line* indirect monitoring of cell growth. Additional molecules are also targeted such as cell growth factors or secreted MMP enzyme secreted.

The second strategy aims at monitoring collagen secretion within the tissue together with cell proliferation. Advantages of Raman spectroscopy is that it allows for a 1cm depth focus of the laser to analyze the internal composition of the tissue. If validated, such strategy could allow for an *on-line* 3D mapping of the cultivated tissue.

4.1.2 Capacitance spectroscopy

Capacitance spectroscopy allows for a non-destructive analysis within 1cm diameter around the probe electrodes. In homogeneous proliferation systems as the dermis porous structures we are currently studying, such technology could allow for monitoring of cell growth. Additionally, as described earlier, capacitance measurement is impacted by charge movement within the cells and the media. Modification of the ECM around the cells with most probably be visible on the permittivity spectra and on the cells characteristic frequency (f_c) values. This technology will also be explored with a dedicated bioreactor specifically design and 3D printed.

4.1.3 Tissue imaging

In-vivo imaging techniques are currently explored to propose an adapted tool for tissue characterization. Here, one of the advantages is to benefit from expertise of scientists used to work with mature tissues. Recent technological advances in the field of optical imaging allow to consider different tools for application to our studies. Different techniques could be envisaged but our main constraint is to select technologies enabling analysis of large tissues and which provide biochemical characterization of deep internal tissue regions.

As example the two-photon microscopy to track cellular activity; the light sheet microscopy coupled with optical clearing, which allow for whole organ imaging and provide overview of tissue changes or the confocal microscopy to interrogate specific regions within the tissue at higher resolution are considered. The most advanced strategy explored in the team is the MRI (Magnetic Resonance Imaging).

MRI (Magnetic Resonance Imaging)

This technology is based on the detection of molecule's protons and is widely used for *in-vivo* or *ex-vivo* organs imaging thanks to MRI scanners. Recently, using BOLD-MRI (Blood oxygen level dependent functional magnetic resonance imaging) a team managed to reach

high imaging resolutions and to detect changes in oxygen supply within mice fetal heart and fetal liver [59].

In our case, the goal is to detect and to monitor the evolution of extracellular matrix production (collagen or elastin secretion, release of the bioink components in the nutritive fluids) as well as the evolution of nutritive fluid (variations in metabolites and oxygen concentrations). Two strategies are explored. First, the off-line analysis of mature large bioprinted tissues within a collaboration with CERMEP platform. Second, the installation of an *on-line* MRI system associated to the cultivation bioreactors. This work is co-developed with Creatis laboratory in Lyon.

6. Scientific production

20 Accepted publications in international journal

Underlined names = supervised student

- P0 In line raman monitoring of metabolic growth inhibitors and amino acids in cho cell culture productions. Emma Petiot, Anita E. Tolouei, Peter G. Slade, Derrick Ruble. *Biotechnology & Bioengineering*. Submitted / under review.
- P1 Advances in Influenza Virus-Like Particles bioprocesses. L. Durous, M. Rosa-Calatrava, E. Petiot. *Expert Review of Vaccine*. Vol 18 (12), p.1285-1300
- P2 SPRi-based hemagglutinin quantitative assay for influenza vaccine production monitoring. L. Durous, T. Julien, B. Padey, A. Traversier, M. Rosa-Calatrava, L. Blum, C. Marquette, E. Petiot. *Vaccine*. Vol 37(12) p.1614-1621.
- P3 Large 3D bioprinted tissue: Heterogeneous perfusion and vascularization. L. Pourchet, E. Petiot, C. Loubière, E. Olmos, M. dos Santos, A. Thépot, L. Blum, C. Marquette. *Bioprinting*. Vol.13, e00039.
- P4 Influenza viruses production : evaluation of a novel avian cell line Duck Cell™-T17. E. Petiot, A. Proust, A. Traversier, L. Durous, F. Dappozze, M. Gras, C. Guillard, J.M. Balloul, M. Rosa-Calatrava. *Vaccine*. Vol 36(22) p.3101-3111.
- P5 Accelerated mass production of influenza virus seed stocks in HEK-293 suspension cell cultures by reverse genetics. E. Milan, T. Julien, R. Baggio, A. Verenero Sanchez, J. Montes, A. Manceur, S. Ansorge, E. Petiot, M. Rosa-Calatrava, A. Kamen. *Vaccine*. Vol 35(26) p.3423-3430.
- P6 Generation of monoclonal pan-hemagglutinin antibodies for the quantification of multiple strains of Influenza. A. Manceur, W. Zou, A. Marcil, E. Paquet, C. Gadoury, B. Jaentschke, X. Li, E. Petiot, Y. Durocher, J. Baardsnes, M. Rosa-Calatrava, S. Ansorge, A. Kamen. *PlosOne*. Vol 12(6). e0180314
- P7 Critical phases of viral production processes monitored by capacitance. E.Petiot, S. Ansorge, M. Rosa-Calatrava, A. Kamen. *Journal of Biotechnology*. Vol 242. p.19-29.
- P8 Particle quantification of influenza viruses by high performance liquid chromatography. J. Transfiguracion, A Manceur, E. Petiot, C.M. Thompson, A. A.Kamen. *Vaccine*. Vol 33(1) p.78-84
- P9 Cellular signaling pathways modulated by influenza infection in HEK293 cells. A. Manceur, A. Kamen, E.Petiot, C.F. Shen, S. Ansorge. *BMC Proceedings*. Vol 9 (9) p.9.

- P10 Critical assessment of influenza VLP production in Sf9 and HEK293 expression systems. C.M. Thompson, E. Petiot, A. Mullick, J. Montes, M. Aucoin, O. Henry, A. Kamen. *BMC Biotechnology*. Vol 15 p. 31.
- P11 Influence of HEK293 metabolism on the production of viral vectors and vaccine. E. Petiot, M. Cuperlovic-Culf · C. F. Shen · A. Kamen. *Vaccine*. Vol 33 (44) p. 5974-81.
- P12 Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. C. Thompson, E. Petiot, A. Lennaertz, O. Henry, A. Kamen. *Virology journal*. Vol 10 p.141.
- P13 Developing a production process for influenza VLPs: a comparison between HEK 293SF and Sf9 production platforms. C.M Thompson, E. Petiot, M. Aucoin, O. Henry, A.A. Kamen. *BMC Proceeding*. Vol 7 (Supp 6) p.22
- P14 Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures. E. Petiot, A. Kamen. *Biotechnol. Prog.* Vol 29(1) p.275-84.
- P15 Metabolic and Kinetic analyses of influenza production in perfusion HEK293 cell culture. E. Petiot, D. Jacob, S. Lanthier, V. Lohr, S. Ansorge, A. Kamen. *BMC Biotechnol.* Vol 11 p. 84.
- P16 Real-time monitoring of adherent Vero cell density and apoptosis in bioreactor processes. E. Petiot, A. el-Wajgali, G. Esteban, C. Gény, H. Pinton, A. Marc. *Cytotechnology*. Vol 64 (4) p.429-461.
- P17 In-situ quantification of microcarrier animal cell culture using near-infrared spectroscopy. E. Petiot, C. Geny, T. Magadoux, H. Pinton, A. Marc. *Process Biochem.* Vol 45 p.1427-1431.
- P18 Kinetic characterization of Vero cell metabolism in a serum-free batch culture process. E. Petiot, F. Blanchard, C. Gény, T. Magadoux, H. Pinton, A. Marc. *Biotech. Bioeng.* Vol 107 (1) p.143-153.
- P19 Rapid screening of serum-free media for the growth of adherent Vero cells by using a small-scale and non-invasive tool. E. Petiot, F. Fournier, C. Gény, H. Pinton, A. Marc. *Appl. Biochem. Biotechnol.* Vol 160 (6) p. 1600-1615.
- P 20 Quality by Design (QbD) for biotechnology-related pharmaceutical. C-F. Mandenius, K. Graumann, T.W. Schultz, A. Premstaller, I-M. Olsson, E. Petiot, C. Clemens, M. Welin. *Biotechnol. J.* Vol 4 (5). p. 600-609.

14 Invited Oral Communications

In blue = in international conferences . Underlined names = supervised student

- O1** New on-line measurements of influenza virus and antigen by SPR - A solution to improve our viral production bioprocesses. Emma Petiot. *Webinar “The analytical VIP”. Horiba company, web. June 2020*
- O2** Bioprinting and Bioprocess. Emma Petiot. *Mc Gill university internal seminar, Division orthopedic surgery. Montréal, Canada February 2020*
- O3** Bioprinting and Bioprocess. Emma Petiot. *Sartorius company internal seminar. Goettingen, Germany. March 2020*
- O4** Bioprocess for human cell & tissue. Emma Petiot, Christophe Marquette. *Agera Lyon. Cellusion, FUJI, TOPPAN, CIRA companies. Tokyo, Japan. November 2019*
- O5** In-line monitoring of Mabs CHO cell culture processes : Application of Raman spectroscopy for metabolic inhibitors monitoring. Emma Petiot. *Conférence Mastère Génie des Procédés Biotechnologiques, June 2019, Lyon, France.*
- O6** Biomaterial properties to protect cells in 3D bioprinting. L.Lemarié, E-J. Courtial, E. Petiot, C.A. Marquette. *5th BIOMAT, Weimar Germany, 2019*
- O7** Quantification at-line et on-line de virus influenza par résonance plasmonique de surface (SPR). Laurent Durous, Christophe Marquette, Emma Petiot. *Société Boeringer Ingelheim, Lyon, France. June 2019*
- O8** In-line monitoring of Mabs CHO cell culture processes: Application of Raman spectroscopy for metabolic inhibitors monitoring. Emma Petiot. *Pfizer US Internal Seminar, Saint Louis, US. May 2019*
- O9** Affinity-based surface plasmon resonance biosensor for in-process influenza hemagglutinin and virus quantification. Laurent Durous, Thomas Julien, Blandine Padey, Aurélien Traversier, Manuel Rosa-Calatrava, Loïc J. Blum, Christophe A. Marquette, Emma Petiot. *2nd ESACT Frontiers Retreat. Zagreb. 2018.*
- O10** Suivi en-ligne et signatures des procédés de production virales. Emma Petiot. *PAT Workshop. Adebitech Paris, France. March 2017*
- O11** Comparison of influenza Virus-Like Particles production processes. Charlotte Froissard, Emma Petiot, Thomas Julien, Manuel Rosa-Calatrava. *1st ESACT Frontiers. Lyon, France 2016.*
- O12** Suivi en-ligne et signatures des procédés de production virales. Emma Petiot. *Session QbD & PAT. Entretien Jacques Cartier, Lyon, France. 2015*

- O13** Near infrared spectroscopy (NIRS) as an in-situ PAT tool to monitor adherent VERO cell culture processes. *E. Petiot, P. Bernard-Moulin, C. Gény, H. Pinton, A. Marc. Workshop "PAT and other recent developments in regulation". 21^{eme} congrés de l'ESACT (European Society of Animal Cell Technology), Dublin, Irlande.2009*
- O14** Evaluation of near infrared (NIR) spectroscopy as PAT tool for adherent VERO cell culture process. *E Petiot, P Bernard-Moulin, C Gény, O Piot, M Manfait, H Pinton, A Marc. Workshop of 7th European Symposium of Biochemical Engineering Science, Faro, Portugal. 2008*

23 Poster in international meetings

- PO1** *26th congrés de l'ESACT (European Society of Animal Cell Technology), Copenhagen, Danemark. 2019*
Cultivation and maturation of large 3D bioprinted tissue in perfusion bioreactors. Emma Petiot, Léa Pourchet, Céline Loubière, Eric Olmos, Laura Chastagnier, Morgan dos Santos, Amélie Thépot, Christophe A. Marquette.
- PO3** *Vaccine Technology VI, Albuferia, Portugal.2016*
Novel avian duckcel-T17 Cell line for production of viral vaccines : Application to influenza viruses production. *E. Petiot, Anais Proust, A. Traversier, L. Durous, F. Dappozze, C. Guillard, J.M. Balloul, M. Rosa-Calatrava.*
- PO4** Pan-HA antibodies for influenza detection and quantification. A. Manceur, A. Marcil, W. Zou, W. Chen, C. Gadoury, E. Petiot, A. Kamen, X. Li, B. Jaentschke, S. Ansoerge.
- PO5** *Cell culture Engineering XIV, Québec, Canada. 2014*
Dielectric spectroscopy as real-time monitoring tool for critical phases of cell viral production. *E. Petiot, Sven Ansoerge, Amine Kamen*
- PO6** *23^{eme} Congrés de l'European Society for animal Cell Technology (ESACT), Lille, France. 2013*
Real-time monitoring of viral production kinetics. E Petiot, S. Ansoerge, A. Kamen.
- PO7** Development of a Production Process for Influenza VLPs: A Comparison of HEK 293SF and Sf9 Production Platforms. *C. Thompson, E. Petiot, A. Mullick, J. Montes, M. Aucoin, O. Henry, A. Kamen.*
- PO8** *Congrès Vaccine Technology IV, Faro, Portugal.*
Influenza production kinetics in HEK293 cell cultures. E Petiot, A. Kamen.
- PO9** Evolution of critical process parameters and operation range for successful scale up and robust manufacturing of REOLYSIN®. S. Ansoerge, J. Transfiguracion, S. Lanthier, R. Voyer, C.F. Shen, A. Bernier, B. Cass, E. Petiot, M. Coffey, A. Kamen.
- PO10** *14th Ontario-Quebec biotechnology conference, Ottawa, Canada*
Development of a Scalable Production Process for Influenza VLPs in cell culture. *C. Thompson, E. Petiot, A. Mullick, J. Montes, M. Aucoin, O. Henry, A. Kamen.*
22^{eme} Congrés de l'European Society for animal Cell Technology (ESACT), Vienne, Autriche. 2011

- PO11** Influenza production in HEK293 cell culture. E. Petiot, A Le Ru, D. Jacob, S. Lanthier, V. Lohr, S. Ansorge, A. Kamen.
- PO12** Real-time monitoring of viral vector and vaccine production processes. S. Ansorge, E. Petiot, A. Kamen.
- PO13** In-situ cell density monitoring and apoptosis detection in adherent Vero cell culture. E. Petiot, A. el-Wajgali, G. Esteban, C. Gény, H. Pinton, A. Marc.
10^{eme} colloque Association Thérapie Génique du Québec, Québec, Canada, 2011
- PO14** Influenza production in HEK293 cell culture. E. Petiot, A Le Ru, D Jacob, S Lanthier, V Lohr, S Ansorge, A Kamen
Congrès Vaccine Technology III, Puerto Vallarta, Mexique. 2010
- PO15** Influence of glucose and glutamine metabolism in animal component-free media for the production of viruses in Vero cells. E. Petiot, C. Gény, F. Blanchard, H. Pinton, A. Marc.
- PO16** HEK293 cells is an efficient platform for large scale manufacturing of influenza vaccine. A. Le Ru, D. Jacob, J. Transfiguracion, S. Ansorge, E. Petiot, O. Henry, A. Kamen.
21^{eme} Congrès de l'European Society for animal Cell Technology (ESACT), Dublin, Irlande. 2009
- PO17** Near infrared spectroscopy as an in-situ PAT tool to monitor adherent VERO cell culture processes. E. Petiot, P. Bernard-Moulin, C. Gény, T. Magadoux, H. Pinton, A. Marc.
- PO18** VERO cell metabolism in animal component-free media: influence of glucose and glutamine substitution. E. Petiot, F. Blanchard, C. Gény, T. Magadoux, H. Pinton, A. Marc.
7th European Symposium of Biochemical Engineering Science (ESBES), Faro, Portugal. 2008
- PO19** Interest of a non-invasive image analyzer to screen culture media in the case of adherent VERO cell cultures. E. Petiot, C. Gény, F. Fournier, H. Pinton, A. Marc.
- PO20** Evaluation of near infrared (NIR) and Raman spectroscopy as PAT tools for adherent VERO cell culture process. E. Petiot, P. Bernard-Moulin, C. Gény, O. Piot, M. Manfait, H. Pinton, A. Marc.
Symposium of productivity and operational excellence in biomanufacturing, Evry, France. 2008
- PO21** Screening of serum-free culture media for adherent VERO cells by a non-invasive method. E. Petiot, C. Gény, F. Fournier, H. Pinton, A. Marc.
- PO22** Culture control of VERO cell on microcarriers by near infrared (NIR) and Raman spectroscopy. E. Petiot, P. Bernard-Moulin, C. Gény, O. Piot, M. Manfait, H. Pinton, A. Marc.
Metabolic Engineering V: Genome to product. Squaw Creek resort, Lake Tahoe, Calif. USA.
- PO23** Insertion of an alternative pathway for succinate production in *Aspergillus niger*. W.A de Jongh, Emma Petiot, Christian Müller, Jens Nielsen.

7. Bibliography

1. Petiot E, Jacob D, Lanthier S, Lohr V, Ansorge S, Kamen AAA, Emma Petiot, Danielle Jacob, Stephane Lanthier, Verena Lohr, Sven Ansorge AK, Petiot E, Jacob D, Lanthier S, et al.: **Metabolic and kinetic analyses of influenza production in perfusion HEK293 cell culture.** *BMC Biotechnol* 2011, **11**:84.
2. Petiot E, Guedon E, Blanchard F, Geny C, Pinton H, Marc A, Gény C, Pinton H, Marc A: **Kinetic characterization of vero cell metabolism in a serum-free batch culture process.** *Biotechnol Bioeng* 2010, **107**:143–153.
3. Petiot E, Proust A, Traversier A, Durous L, Dappozze F, Gras M, Guillard C, Balloul J-M, Rosa-Calatrava M: **Influenza viruses production: Evaluation of a novel avian cell line DuckCelt®-T17.** *Vaccine* 2017, doi:10.1016/j.vaccine.2017.03.102.
4. Takizawa T, Matsukawa S, Higuchi Y, Nakamura S, Nakanishi Y, Fukuda R: **Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells.** *J Gen Virol* 1993, **74**:2347–55.
5. McSharry JJ: **Analysis of virus-infected cells by flow cytometry.** *Methods* 2000, **21**:249–57.
6. Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D: **Apoptosis: a mechanism of cell killing by influenza A and B viruses.** *J Virol* 1994, **68**:3667–73.
7. Schulze-Horsel J, Schulze M, Agalaridis G, Genzel Y, Reichl U: **Infection dynamics and virus-induced apoptosis in cell culture-based influenza vaccine production – Flow cytometry and mathematical modeling.** *Vaccine* 2009, **27**:2712–2722.
8. Lohr V, Rath a, Genzel Y, Jordan I, Sandig V, Reichl U: **New avian suspension cell lines provide production of influenza virus and MVA in serum-free media: studies on growth, metabolism and virus propagation.** *Vaccine* 2009, **27**:4975–82.
9. Terrier O, Marcel V, Cartet G, Lane DP, Lina B, Rosa-Calatrava M, Bourdon J-C: **Influenza A viruses control expression of proviral human p53 isoforms p53 β and Delta133p53 α .** *J Virol* 2012, **86**:8452–60.
10. Zhao M, Wang L, Li S: **Influenza A virus-host protein interactions control viral pathogenesis.** *Int J Mol Sci* 2017, **18**:1–15.
11. Manceur AP, Kamen AA, Petiot E, Shen CF, Ansorge S, A. Manceur, A. Kamen, E. Petiot, C.F. Shen SA: **Cellular signaling pathways modulated by influenza infection in HEK293 cells.** *BMC Proc* 2015, **9**:P9.
12. Bédard C, Kamen A, Tom R, Massie B: **Maximization of recombinant protein yield in the insect cell/baculovirus system by one-time addition of nutrients to high-density batch cultures.** *Cytotechnology* 1994, **15**:129–138.
13. Petiot E, Cuperlovic-Culf M, Shen CFCF, Kamen A: **Influence of HEK293 metabolism on the production of viral vectors and vaccine.** *Vaccine* 2015, **33**:5974–5981.
14. Nikolay A, Léon A, Schwamborn K, Genzel Y, Reichl U: **Process intensification**

- of EB66® cell cultivations leads to high-yield yellow fever and Zika virus production.** *Appl Microbiol Biotechnol* 2018, **102**:8725–8737.
15. Carinhas N, Bernal V, Yokomizo AY, Carrondo MJT, Oliveira R, Alves PM: **Baculovirus production for gene therapy: the role of cell density, multiplicity of infection and medium exchange.** *Appl Microbiol Biotechnol* 2009, **81**:1041–9.
 16. Dill V, Ehret J, Zimmer A, Beer M, Eschbaumer M: **Cell density effects in different cell culture media and their impact on the propagation of foot-and-mouth disease virus.** *Viruses* 2019, **11**.
 17. Petiot E, Fournier F, Gény C, Pinton H, Marc A, Geny C, Pinton H, Marc A, Gény C: **Rapid screening of serum-free media for the growth of adherent Vero cells by using a small-scale and non-invasive tool.** *Appl Biochem Biotechnol* 2010, **160**:1600–1615.
 18. Mulukutla BC, Mitchell J, Geoffroy P, Harrington C, Krishnan M, Kalomeris T, Morris C, Zhang L, Pegman P, Hiller GW: **Metabolic engineering of Chinese hamster ovary cells towards reduced biosynthesis and accumulation of novel growth inhibitors in fed-batch cultures.** *Metab Eng* 2019, **54**:54–68.
 19. Christie A, Butler M: **Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase.** *J Biotechnol* 1994, **37**:277–290.
 20. Petiot E, Kamen AA: **Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures.** *Biotechnol Prog* 2012, **29**:275–284.
 21. Petiot E, Proust A, Traversier A, Durous L, Dappozze FF, Gras M, Guillard C, Balloul J-M, Rosa-Calatrava M: **Influenza viruses production: Evaluation of a novel avian cell line DuckCelt®-T17.** *Vaccine* 2018, **36**:3101–3111.
 22. Le Ru A, Jacob D, Transfiguracion J, Ansorge S, Henry O, Kamen AA: **Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing.** *Vaccine* 2010, **28**:3661–3671.
 23. Trebbien R, Larsen LE, Viuff BM: **Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs.** *Virol J* 2011, **8**:1–14.
 24. Thompson CMCM, Petiot E, Lennaertz A, Henry O, Kamen AAA: **Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches.** *Virol J* 2013, **10**:1–14.
 25. Mandenius C-F, Graumann K, Schultz TW, Premstaller A, Olsson I-M, Petiot E, Clemens C, Welin M: **Quality by design (QbD) for biotechnology-related pharmaceuticals.** *Biotechnol J* 2009, **4**:600.
 26. Petiot E, El-Wajgali A, Esteban G, Gény C, Pinton HHH, Marc A, Wajgali A El, Esteban G, Geny C, Pinton HHH, et al.: **Real-time monitoring of adherent Vero cell density and apoptosis in bioreactor processes.** *Cytotechnology* 2012, **64**.
 27. Petiot E, El-Wajgali A, Esteban G, Geny C, Pinton HH, Marc A, Gény C, Pinton HH, Marc A: **In-situ cell density monitoring and apoptosis detection in adherent Vero cell bioreactor cultures.** *BMC Proc* 2011, **5**:P8.
 28. Opel CF, Li J, Amanullah A: **Quantitative modeling of viable cell density, cell size, intracellular conductivity, and membrane capacitance in batch**

- and fed-batch CHO processes using dielectric spectroscopy.** *Biotechnol Prog* 2010, **26**:1187–99.
29. Petiot E, Ansorge S, Rosa-Calatrava M, Kamen A: **Critical phases of viral production processes monitored by capacitance.** *J Biotechnol* 2017, **242**.
 30. Ma F, Zhang A, Chang D, Velev OD, Wiltberger K, Kshirsagar R: **Real-time monitoring and control of CHO cell apoptosis by in situ multifrequency scanning dielectric spectroscopy.** *Process Biochem* 2019, **80**:138–145.
 31. Petiot E, Bernard-Moulin P, Magadoux T, Gény C, Pinton H, Marc A: **In situ quantification of microcarrier animal cell cultures using near-infrared spectroscopy.** *Process Biochem* 2010, **45**:1427–1431.
 32. Patel BA, Pinto NDS, Gospodarek A, Kilgore B, Goswami K, Napoli WN, Desai J, Heo JH, Panzera D, Pollard D, et al.: **On-Line Ion Exchange Liquid Chromatography as a Process Analytical Technology for Monoclonal Antibody Characterization in Continuous Bioprocessing.** *Anal Chem* 2017, **89**:11357–11365.
 33. Li M, Ebel B, Chauchard F, Guédon E, Marc A: **Parallel comparison of in situ Raman and NIR spectroscopies to simultaneously measure multiple variables toward real-time monitoring of CHO cell bioreactor cultures.** *Biochem Eng J* 2018, **137**:205–213.
 34. Saint L, Gaillard S, Devos O, André S, Cristau L Saint, Gaillard S, Devos O, Calvosa E, Duponchel L: **In-line and real-time prediction of recombinant antibody titer by in situ Raman spectroscopy.** *Anal Chim Acta* 2015, **892**:148–152.
 35. Transfiguracion J, Manceur AP, Petiot E, Thompson CM, Kamen AA: **Particle quantification of influenza viruses by high performance liquid chromatography.** *Vaccine* 2015, **33**.
 36. Moulès V, Terrier O, Yver M, Riteau B, Moriscot C, Ferraris O, Julien T, Giudice E, Rolland J-P, Erny A, et al.: **Importance of viral genomic composition in modulating glycoprotein content on the surface of influenza virus particles.** *Virology* 2011, **414**:51–62.
 37. Transfiguracion J, Manceur AP, Petiot E, Thompson CM, Kamen AAA: **Particle quantification of influenza viruses by high performance liquid chromatography.** *Vaccine* 2015, **33**:78–84.
 38. Manceur AP, Zou W, Marcil A, Paquet E, Gadoury C, Jaentschke B, Li X, Petiot E, Durocher Y, Baardsnes J, et al.: **Generation of monoclonal pan-hemagglutinin antibodies for the quantification of multiple strains of influenza.** *PLoS One* 2017, **12**:e0180314.
 39. Chang YF, Wang WH, Hong YW, Yuan RY, Chen KH, Huang YW, Lu PL, Chen YH, Chen YMA, Su LC, et al.: **Simple Strategy for Rapid and Sensitive Detection of Avian Influenza A H7N9 Virus Based on Intensity-Modulated SPR Biosensor and New Generated Antibody.** *Anal Chem* 2018, **90**:1861–1869.
 40. Durous L, Julien T, Padey B, Traversier A, Rosa-Calatrava M, Blum LJ, Marquette CA, Petiot E: **SPRi-based hemagglutinin quantitative assay for influenza vaccine production monitoring.** *Vaccine* 2019, **37**:1614–1621.
 41. Ansorge S, Lanthier S, Transfiguracion J, Henry O, Kamen A: **Monitoring**

- lentiviral vector production kinetics using online permittivity measurements. *Biochem Eng J* 2011, **54**:16–25.
42. Genzel Y, Reichl U: **Continuous cell lines as a production system for influenza vaccines.** *Expert Rev Vaccines* 2009, **8**:1681–1692.
 43. Genzel Y, Lohr V, Behrendt I, Scharfenberg K, Kamen A, Schiedner G, Jordan I, Reichl U, Gmbh CP: *Cell culture-based influenza virus production : suspension or adherent producer cell lines ?* 2012.
 44. Kang S-M, Song J-M, Quan F-S, Compans RW: **Influenza vaccines based on virus-like particles.** *Virus Res* 2009, **143**:140–6.
 45. Mair CM, Ludwig K, Herrmann A, Sieben C: **Receptor binding and pH stability - How influenza A virus hemagglutinin affects host-specific virus infection.** *Biochim Biophys Acta - Biomembr* 2014, **1838**:1153–1168.
 46. Milián E, Kamen AA: **Current and Emerging Cell Culture Manufacturing Technologies for Influenza Vaccines.** 2015, **2015**.
 47. Milián E, Julien T, Biaggio R, Venereo-Sanchez A, Montes J, Manceur AP, Ansorge S, Petiot E, Rosa-Calatrava M, Kamen A: **Accelerated mass production of influenza virus seed stocks in HEK-293 suspension cell cultures by reverse genetics.** *Vaccine* 2017, **35**.
 48. Molinas M de los MB: **Transient Transfection of HEK293 Cells in Suspension : Process Characterization and Optimization by Applying Invasive Nucleotide and Non-invasive Electronic Nose Technology.** 2005,
 49. Thompson CM, Petiot E, Aucoin MG, Henry O, Kamen AA: **Developing a production process for influenza VLPs: a comparison between HEK 293SF and Sf9 production platforms.** *BMC Proc* 2013, **7**:P22.
 50. Thompson CMM, Petiot E, Mullick A, Aucoin MGM, Henry O, Kamen AAA: **Critical assessment of influenza VLP production in Sf9 and HEK293 expression systems.** *BMC Biotechnol* 2015, **15**:31.
 51. Venereo-Sanchez A, Gilbert R, Simoneau M, Caron A, Chahal P, Chen W, Ansorge S, Li X, Henry O, Kamen A: **Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate.** *Vaccine* 2016, doi:10.1016/j.vaccine.2016.04.089.
 52. Durous L, Rosa-Calatrava M, Petiot E: **Advances in influenza virus-like particles bioprocesses.** *Expert Rev Vaccines* 2019, **18**:1285–1300.
 53. Pourchet LJ, Thepot A, Albouy M, Courtial EJ, Boher A, Blum LJ, Marquette CA: **Human Skin 3D Bioprinting Using Scaffold-Free Approach.** *Adv Healthc Mater* 2016, doi:10.1002/adhm.201601101.
 54. Masaeli E, Forster V, Picaud S, Karamali F, Nasr-Esfahani MH, Marquette C: **Tissue engineering of retina through high resolution 3-dimensional inkjet bioprinting.** *Biofabrication* 2020,
 55. Pourchet L, Petiot E, Loubière C, Olmos E, Dos Santos M, Thépot A, Loïc BJ, Marquette CA: **Large 3D bioprinted tissue: Heterogeneous perfusion and vascularization.** *Bioprinting* 2019, **13**:1–7.
 56. Dong J, Opresko LK, Dempsey PJ, Lauffenburger DA, Coffey RJ, Wiley HS: **Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor.** *Proc Natl Acad Sci U S A* 1999, **96**:6235–6240.

57. Zohar B, Blinder Y, Mooney DJ, Levenberg S: **Flow-Induced Vascular Network Formation and Maturation in Three-Dimensional Engineered Tissue.** *ACS Biomater Sci Eng* 2018, **4**:1265–1271.
58. Plunkett N, O'Brien FJ: **Bioreactors in tissue engineering.** *Technol Heal Care* 2011, **19**:55–69.
59. Shapiro J, Ginosar Y, Gielchinsky Y, Elchalal U, Bromberg Z, Corchia-Nachmanson N, Abramovitch R: **BOLD-MRI demonstrates acute placental and fetal organ hypoperfusion with fetal brain sparing in response to phenylephrine but not ephedrine.** *Placenta* 2020, **90**:52–57.