Grant Agreement no. 317916

LiPhos

Living Photonics: Monitoring light propagation through cells

INSTRUMENT: Collaborative Project (Small or Medium Scale Focused Research Project)

OBJECTIVE: ICT-2011.3.5

D1.1: LiPhos Specifications

Due Date of Deliverable: 30th April 2013
Completion Date of Deliverable: 9th May 2013

Start date of project: 1st November 2012 Duration: 36 months

Lead partner for deliverable: Cellix

Revision: v2.0

Project co-funded by the European Commission within the 7th Framework Programme (2007-2013)		
Dissemination Level		
PU	Public	✓
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including Commission Services)	
CO	Confidential, only for members of the consortium (including Commission Services)	

Document History

Issue Date	Version	Changes Made / Reason for this Issue
13th December 2012	v0.1	First draft
29th April 2013	v1.1	Second draft
7th May 2013	v1.2	Minor additions from all partners
9th May 2013	v2.0	Final version

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1. Introduction

Deliverable D1.1 sets out the foundation for the entire project, taking input from all partners. Here we outline the specifications for each part of the project, which includes determining:

- User requirements
- Target cell types, reagents and culture parameters to be used
- Requirements of the photonic Lab-on-the-chip:
    - microfluidic elements
    - materials
    - pumping solutions
    - integration with detection and incubation system
- Protocols for laboratory evaluation of the photonic Lab-on-the-chip

WP1 addresses the overall LiPhos system. As such, it is not the purpose of WP1 to give detailed specifications of features and parameters that can better be specified in other work packages. Therefore, more detailed specifications will have to be developed within each of the technical work packages, as well as specifications to define interfaces between components.

2. LiPhos System Requirements: Targets and Specifications

2.1. Project overall goal

The LiPhos project targets the development of innovative biophotonic diagnosis methods and tools, which are implemented using cells as constituent material. In this approach the light remains confined in a waveguide whose core is uniquely composed of cells. The cells play a two-fold role: i) they form the biomaterial with higher refractive index than the surrounding media, thus defining the waveguide; ii) they are interrogated by the light coupled into them, acting as reporter elements and exhibiting a specific spectral response. The advantage of this configuration is the highly efficient cell-light interaction, making it possible to diagnose diseases by measuring and comparing their photonic fingerprint (PIN). This key parameter, newly introduced in LiPhos, consists of the spectral response of the living photonics and includes the different inherent or acquired bands and peaks (scattering, absorbance and/or fluorescence) directly related to the cell culture under study.

The overall aim of the project is to investigate photonic fingerprints (PIN) by measuring adherent cell layers cultured under controlled conditions and representing diseased or healthy states and to apply PIN information in the diagnosis of cardiovascular diseases (CVD).

2.2. User requirements

The first-generation LiPhos system, according to end-users RUG, AU, and CNIC, is one which is modular, robust, reliable and user-friendly.

1. **It allows easy, reproducible seeding and culturing in small channels** or chambers of endothelial and/or vascular smooth muscle cells and macrophages, or culture of bio-artificial or patient arterial segments.
2. **It has the capability to run up to four parallel experiments**, with an optimum of six parallel experiments in the final system.
    - Parallel experiments could refer to either simultaneous experiments with two different cell types or two different flow and/or medium conditions. Six is an optimum number, as this allows two different experiments done in triplicate, thus increasing the possibility of obtaining statistically significant data.

3. **Experimental flexibility** with respect to perfusion of cells or arterial segments is required, with a built-in capability to switch between (at least) two different media for each culture.
4. Photonics and/or microscope imaging should be possible through use of a simple, robust plug-in optical or **microscope interface**.

The microfluidic system:

a. Should include a **4-to-6 channel (chamber) microfluidic culture device**, **pump(s) and valves** as necessary to ensure the experimental flexibility described above, and leak-free interface between medium reservoirs and pump and the device.
b. **Protocols for seeding cells should be easy to learn and reproducible** using the microfluidic system and pump(s) provided.
c. The microfluidic module with associated pumps and valves should **fit in a standard incubator** to benefit from a controlled atmosphere of 95% air and 5% carbon dioxide.

Photonic measurements:

a. **Can be made outside the incubator** – Maintaining the cultures at a temperature of 37°C can be achieved through use of a heating platform (standard for microscope stages and most likely applicable to the photonic measurements here as well.
b. **The optical detection interface should be simple to use** ("plug-and-play") and robust enough that no alignment of detection components with the microfluidic module is necessary. Alternatively, alignment is achieved within a few minutes in an automated manner.

The user requirements have been summarised in Table 1.

Table 1: Summary of user requirements for LiPhos system.

Liphos system and experimental requirements	Microfluidic biochip	Photonics
Easy and reproducible seeding of cells within microchannels	4-6 microfluidic channels on the chip	Optical and photonic system can be outside the incubator
4-6 channels perfused in parallel	Easy access for seeding cells in microchannels	Simple optical detection interface
Placed inside the incubator, with pump and two culture medium bottles	Easy connection to two pumps, pumping at least two culture medium types	Two options: either no alignment of the optical components is necessary or alignment is done in an automated manner
Flexible experimental conditions. Two different culture media perfused or one medium at two different flow rates (two independent pumps).		
Heated microscopy frame to maintain 37^{o}C during optical observation.		

2.3. Cellular, chemical and physical parameters to be monitored

In this part of specification we have identified primary cells / cell lines, which will be used for validation of the photonic lab-on-a-chip system and applied in evaluation of cardiovascular disease progression.

To date, several primary cells of human and mouse origin have been successfully used in partner institutions (RUG, CNIC, AU, Cellix) and corresponding reagents, specifications and protocols are covered in this part of specification (described also in Appendix A). Some primary cell cultures (aortic endothelial cells) are very suitable for studies of cardiovascular disease and atherosclerosis, in particular.

Table 2: Cells to be used in photonic cell culture experiments.

Cells	Acronym	Primary	Supplier	Supplier Product Name	Supplier Product Code	Passage Number
Human Umbilical Vein Endothelial Cells	HuVEC	Yes	Promocell	HuVEC pooled proliferating	C-12253	3-5
Human Umbilical Vein Endothelial Cells	HUVEC	Yes	University Medical Center Groningen HUVEC Facility	HuVEC pooled proliferating	N/A	3-5
Mouse microvascular endothelial cells from kidney	mKECs	Yes	Central Animal Facility – University of Groningen	N/A	N/A	3-5
Mouse endothelial cells from aorta	mAECs	Yes	CNIC	N/A	N/A	1-2
Mouse Smooth Muscle Cells from aorta	mASMCs	Yes	CNIC	N/A	N/A	1-2
Mouse Bone Marrow-Derived Macrophages	mBMDM	Yes	CNIC	N/A	N/A	1-2
Mouse peritoneal macrophages	mPM	Yes	CNIC	N/A	N/A	1-2

Table 3: Reagents to be used in conjunction with cell seeding and culture.

Reagents	Acronym	Supplier	Supplier Product Name	Supplier Product Code
Fibronectin		R&D Systems	Human Fibronectin, CF 2mg	1918-FN-02M
Culture Media		Promocell	Endothelial Medium Kit	C22110
Murine interferon γ	IFNγ	Peprotech	Murine interferon γ	315-05
Murine interleukine 4	IL-4	R&D Systems	Murine interleukine 4	404-ML
Lipopolysaccaride	LPS	Sigma-Aldrich	Lipopolysaccaride	L2654,
Recombinant murine macrophage-colony stimulating factor	M-CSF	Peprotech	Murine M-CSF	315-02
Endothelial Cell Growth Supplement	ECGS	BD Pharmingen	Endothelial Cell Growth Supplement	554006
Heparin sodium salt from porcine intestinal mucosa	Heparin	Sigma-Aldrich	Heparin sodium salt from porcine intestinal mucosa	H3393
Dynabeads Sheep anti-Rat IgG	Dynabeads	Life Technology	Dynabeads Sheep anti-Rat IgG	110.35
Purified rat Anti-Mouse CD102 Monoclonal Antibody	anti-anti-mouse CD102	BD Pharmigen	Purified Rat Anti-Mouse CD102	553326
Collagen from calf skin	collagen	Sigma-Aldrich	Collagen from calf skin	C8919
Gelatin from bovine skin	gelatin	Sigma-Aldrich	Gelatin from bovine skin	G9382
Tumour Necrosis Factor α	TNF α	Boehringer-Ingelheim		
Collagenase type 2	Collagenase II	Worthington Biochemical Corporation	CLS-2 LS004176	Collagenase type 2, 300 U/mg

2.4. Photonic requirements

We have summarised optical equipment parameters, fibre optics and necessary tools for the photonic lab-on-the-chip experiments. Several institutions (RUG, CLX, CNIC) have already purchased similar spectrometers, light sources and optical fibres, and are setup to run photonic experiments which can be easily correlated.

Table 4: Parameters of optical equipment and components.

Light source	**Wavelength range (µm)**	**Nominal output power (Watt)**	**Stabilization time (min)**	**Bulb lifetime (h)**	**Fibre optic connector**	**Supplier**	**Notes**
	0.36-2.00	7	5	10.000	SMA	Ocean Optics	HL-2000-FHSA-LL
Spectro-meter	**Wavelength range (µm)**	**Peak QE**	**Integration time (sec)**	**Dynamic range typ.**	**Signal-to-noise ratio (SNR)**	**Supplier**	**Notes**
	0.2-1.1	90%	0.008-900	25000:1	1000:1	Ocean Optics	QE65 Pro
Fibre optics	**Wavelength Range (µm)**	**Core Diameter (µm)**	**NA**	**Cladding Diameter (µm)**	**Jacket**	**Supplier**	**Notes**
	0.35-2.2	200 ± 8	0.22	240 ± 5	FT030	Thorlabs	SMA-SMA 5 meter fiber optics FG200LCC
Fibre stripper						**Supplier**	**Notes**
						Thorlabs	T12S16 - Fibre Stripping Tool (Clad/Coat: 230/400 µm)
Fibre cleaver						**Supplier**	**Notes**
						Thorlabs	S90R -Ruby DualScribe™ Fibre Optic Scribe
Fibre Optics	**Wavelength Range (µm)**	**Core Diameter (µm)**	**NA**	**Cladding Diameter (µm)**	**Jacket**	**Supplier**	**Notes**
	0.4 – 2.4	50 ± 2%	0.22	125 +1/-3	FT030	Thorlabs	AFS50/125Y fibre optics (Custom SMA – flat cleave end for SCA)
	0.4 – 2.4	105 ± 2%	0.22	125 +1/-3	FT030	Thorlabs	AFS105/125Y fibre optics (Custom SMA – flat cleave end for SCA)
Fibre Stripper						**Supplier**	**Notes**
						Thorlabs	T08S13 Clad/Coat 125/250µm

2.5. Microfluidics and integration requirements

We have summarised specifications of three biochip designs according to three configurations for PIN detection in a single layer of cells (Single Layer Living Photonics, SLLP), from multiple layers of cells (Multiple Layer Living Photonics, MLLP), and from single cells (Single Cell Analysis, SCA). The main parameters of the specification are chip footprint, dimensions of the biochip, material of the bottom coverslip and base of the biochip, and the number of channels. We have also evaluated how the three approaches or configurations can be applied, along with their advantages and disadvantages.

2.5.1. Single Layer Living Photonics (SLLP) configuration biochip

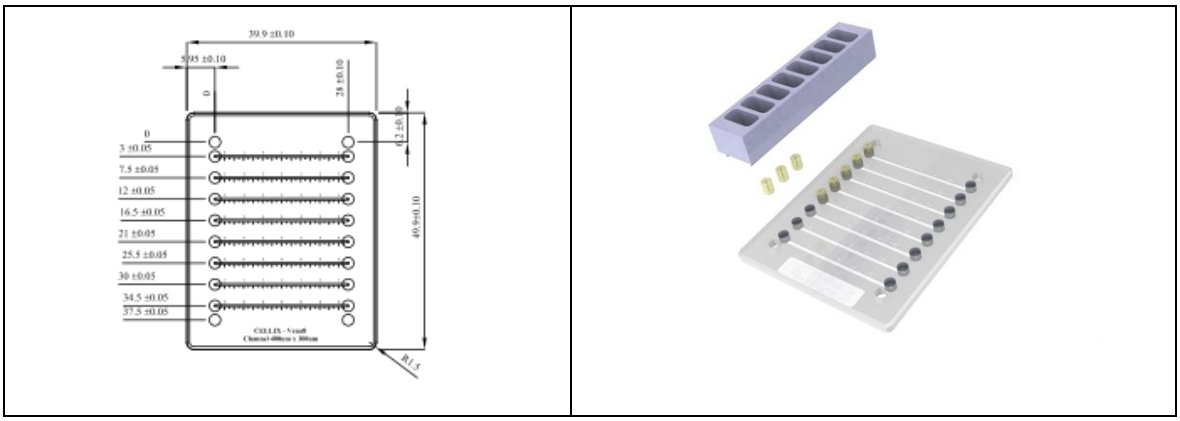
Applications: Comparison of healthy versus diseased cells grown in appropriate physiological conditions.

Advantages: Easy to handle cells, high optical signal, allows study of complex optical/photonic/PIN parameters, study of physical (flow) and chemical compounds on cells, high throughput.

Other possible advantages: Coupling of light into cell layer facilitated, high optical signal.

Disadvantages: Altered cell properties e.g. expression and physiological function compared to multilayer culture approach and cells in tissue.

Chip footprint (example):



Overall biochip dimensions: 40 mm x 50 mm

This is the size of Cellix's standard Vena8 biochips. Cellix already has a variety of chip holders to integrate with different microscope types (Zeiss, Nikon, Olympus etc.).

Biochip substrate / waveguide part of biochip

Dimensions: 40 mm x 50 mm **with possibility to decrease size - increase number of waveguide parts on the wafer**. Size of optical part of chip is limited by size of wafer and cleanroom processes. Substrates are 100 mm diameter with uniform processing area of about 90 mm. Machinery for 150 mm wafers is available, but has not been tested for use to fabricate optical structures. Smaller size is usually preferred as more chips fit on one wafer (more experiments per fabrication run).

Material: Fused silica glass. Material of the waveguide is Silicon Nitride.

Thickness: 0.17 mm – 0.5 mm. Thinner substrates would allow observation using short working distance and high numerical aperture microscope objectives.

Biochip base / microchannel and fluidic port part

Dimensions: 40 mm x 50 mm

Material: Acrylic

Thickness: 2.5 mm

Number of Microchannels: 4 – 8 (to address user requirements).

Microchannels spacing: 4.5 mm

Microchannel dimensions: 800 µm width x 120 µm depth. Minimal microchannel depth is 5 µm, maximal is 160 µm. Minimal microchannel width is 25 µm, maximal is 3.5 mm.

Microchannels input/output ports: See Appendix D for drawings.

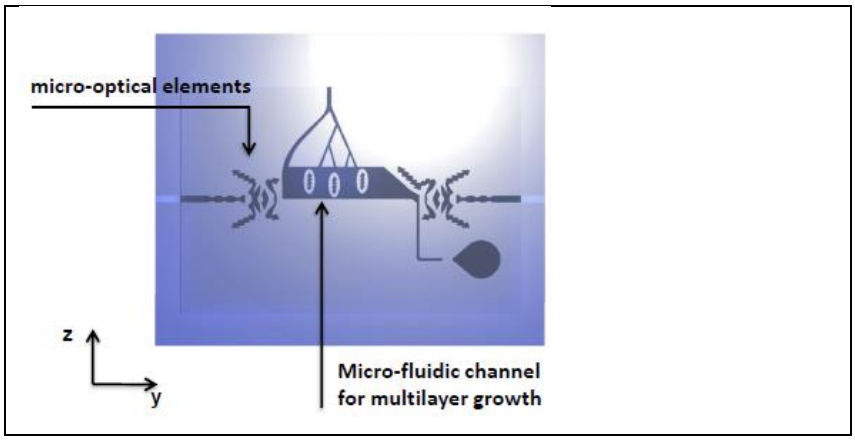
2.5.2. Multiple Layer Living Photonics (MLLP) configuration biochip

Applications: Mainly to provide benchmarking, pharmacological testing of PIN parameters.

Advantages: Multilayer preparations are already established, closer to *in vivo* conditions, complex PIN.

Disadvantages: Difficult-to-interpret PIN due to the system complexity, low signal to noise ratio, low throughput.

Chip footprint (example):



Overall biochip dimensions: 40 mm x 50 mm

Biochip substrate / optical part of biochip

Dimensions: 40 mm x 50 mm

Material: PDMS. Material of the waveguide (optics) is PDMS.

Thickness: 2.5mm

Biochip base / microchannel and fluidic port part

Dimensions: same as above.

Material: PDMS

Thickness: 2.5 mm

Number of microchannels: 2 – 4 (to address user requirements).

Microchannel dimensions: 800 µm width x 120 µm depth.

Microchannels input/output ports: see Appendix D for drawings.

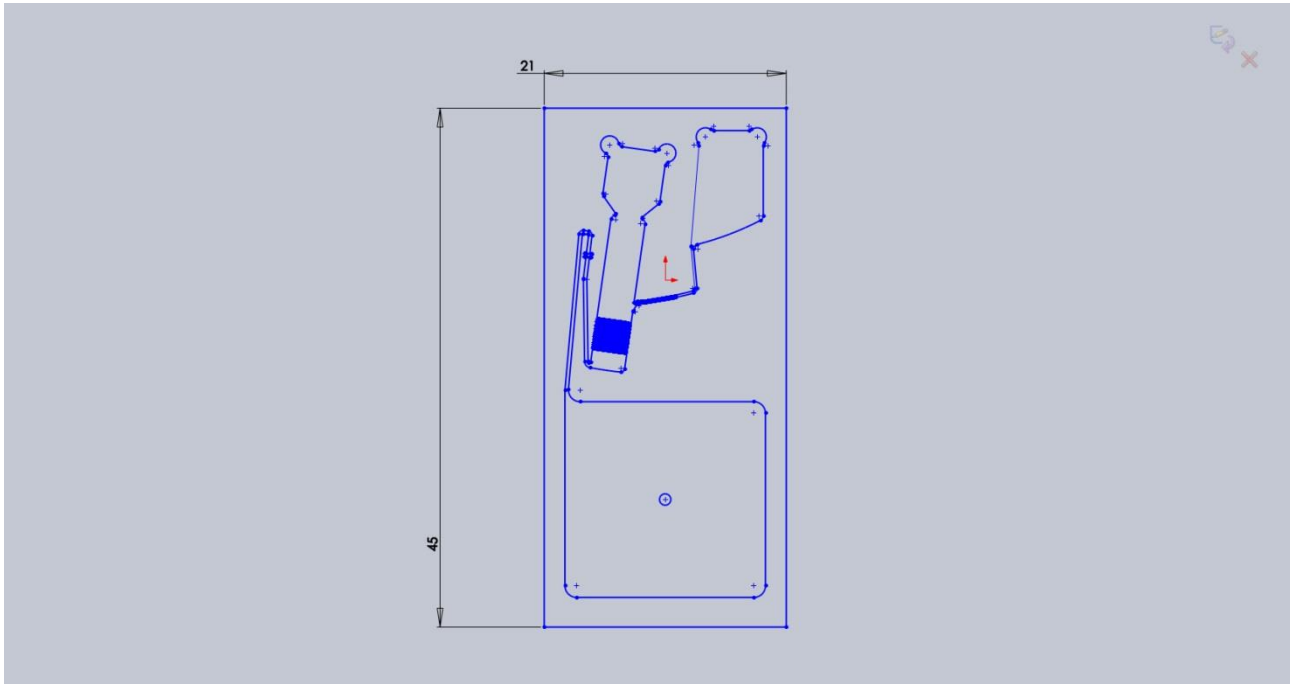
2.5.3. Single Cell Analysis (SCA) configuration biochip

Applications: Endothelial progenitor cells, macrophages.

Advantages: High throughput, PIN limited to single cells which will allow measurements reflecting properties of the cell, PIN probably easier to analyze providing larger signal strength when healthy versus non-healthy cells are compared.

Disadvantages: Small signal compared to layer of cells, altered properties of cells with no relation to *in situ* cells in the vasculature, no connection of analysed cell with other cells, less organisation of cell cytoskeleton.

Chip footprint – layout of PDMS single cell V cup biochip



Overall Biochip Dimensions: 40 mm x 60 mm

DCU have developed a non-standard size chip, which can capture single cells and position the cells (via laser tweezers) for optical analysis and PIN readout. The chip is utilized on a centrifugal platform and cells are captured using V cup structures fabricated on the chip. The chip also features trap locations where single cells can be moved for PIN readout (via laser tweezers).

Biochip substrate / waveguide art of biochip

Dimensions: 21 mm x 45 mm

Material: coverslip made of borosilicate glass.

Thickness: 0.13 – 0.16 mm. Thinner substrates would allow observation using short working distance and high numerical aperture microscope objectives.

Biochip support holder / middle layer

Dimensions: 40 mm x 60 mm

Material: PMMA

Thickness: 1.5mm

Biochip base / microchannel and fluidic port part

Dimensions: 21 mm x 45 mm

Material: PDMS

Thickness: 2.5 mm

Number of Vcups: V cups are arranged in a staggered array of 47 x 24 (w x l) cups, allowing for a total of 1128 individual cells to be trapped on the V cup array. 16 pillar based locations are also on the biochip to facilitate a sub-population of cells to be selected (via laser tweezers) and have further single cell assays performed.

2.6. Fluidic and integration requirements

This part of specification relates to fluidic and integration requirements such as the following: microscope frame integration, photonics integration, and fluidic pumping specifications. These specifications are directly related to the **user requirements** and are **not finalized yet** as it is necessary to complete designs and drawings in order to have better estimation of space required for integrated device/ holder/biochip. Details of the chip holder dimensions given below are therefore only estimates at this stage.

Table 5: Fluidic interface and integration requirements.

Device Placement	Inside the incubator
Chip holder dimensions	This will be dependent on the chip footprint. Currently available microscope frames through Cellix 120 mm x100 mm for range of microscopes (Zeiss, Nikon, Olympus). These dimensions will increase as pumps and media bottles needs to be integrated onto the frame (extra space requirement: 40 mm x 40 mm). LioniX needs to be able to access at least one edge of the optical chip to be able to couple light into the waveguides. Not sure how this might conflict with current chip holder solutions. Typically, a Fiber Alignment Unit (FAU) is cemented to the side of the optical chip. Work will also be done an automated alignment procedure. The extra space requirements for this are foreseen to stay within 15 mm x 100 mm x 50 mm.
Pump Fluidics	Cellix dual Kima pump for single layer and multi-layer approach and Cetoni Nemesys multichannel syringe pump for single cell approach.
Number of independent channels	2
Media bottles	2
Flow rate	µL/min range
Dead volumes	600 µl
Flow direction	Infuse only Kima pump, Infuse and withdraw modes Cetoni Nemesys.
Media Recirculation	Kima pump recirculation of culture media.

3. Conclusions

In this specification we have summarised key user requirements for the LiPhos cell culture and observation system and have identified cellular, chemical and photonics parameters, microfluidic biochip specifications and system integration specifications in order to fulfil the requirements set by the end-users within the scope of LiPhos project. Several biochip configurations have been proposed to address three approaches within LiPhos: detection of PIN from a single layer, multi-layer and single cells. The designs and specifications of biochips are not yet finalised, as more work is required to successfully integrate microfluidic channel components with optical waveguides, optical fibres and also with microscope frames and pumping. This will evolve throughout the project and changes will be reflected in the subsequent modifications and revisions of D1.1.

Appendix A – *Supplier Documentation related to Cell Types and Culture*

All partners submitted the supplier documentation related to cell types used including preparation methods. If a partner follows a different protocol (even minor tweaks) compared to that recommended by the supplier for cell preparation, culture etc., this absolutely must be indicated here. As far as possible, all partners should follow the same protocols at every stage to ensure that data can be easily compared across multiple partner sites.

Isolation of mouse aortic endothelial cells (mAEC) (CNIC)

1. Remove aortas from 5-7 mice (20-25 g body weight) and place them in cold PBS (Falcon 50 mL tube).
2. Always work in sterile conditions from this step (cabinet). Wash aortas with PBS once.
3. Remove the fat around aorta.
4. Incubate the aortas with 0.65 mg/ml Collagenase type 2 (300 U/mg CLS-2 LS004176-Worthington Biochemical Corporation) in DMEM medium (Dulbecco's Modified Eagle's Medium - high glucose D5796, supplemented with 1% Penincilin/Streptomycine) for 20´ at 37 ºC.
5. Remove the adventitia from aorta in culture media.
6. Cut the aortas in rings with a sterile scalpel and put them in DMEM containing 4 mg/ml of Collagenase at 37 ºC for 40 min. Stop reaction by adding DMEM enriched with 10% FBS.
7. Pellet the aortic rings (900 rpm 4 min.) and wash 2-3 times with DMEM 10% FBS. Discard the supernatant.
8. Dissolve pellet and culture the digested portions in a p60 plate previously coated with 0.5% sterile gelatin (Gelatin from bovine skin G9382 Sigma-Aldrich) and 0.1 mg/ml collagen (Collagen from calf skin Ref C8919 Sigma-Aldrich) with DMEM-FBS media.
9. After 2 days of incubation, several cell colonies should be observed. Remove medium and wash cells 2-3 times with PBS.
10. Incubate the cells with EC medium (DMEM:F12, 1:1 Mixture 12-719F – Lonza supplemented with 1% Penincilin/Streptomycine, Glutamine, 10 mM Hepes and Fungizone) containing CD102 antibody (3.5 µl/ml, Purified Rat Anti-Mouse CD102 - ICAM-2 Monoclonal Antibody – BD Pharmingen) for 30´ at 4º C with constant movement. After antibody incubation, remove the medium, wash the cells 2-3 times with PBS and incubate them with secondary antibody associated with magnetic beads (Dynabeads® Sheep anti-Rat IgG Ref 110.35, Invitrogen) in EC media for 30´ at 4º C and with movement.
11. Wash the cells with PBS (2-3 times) and detach them with trypsin. Stop the trypsin activity with DMEM-FBS and directly sort the cells with the magnetic platform (DynaMag™-15 Magnet – Life technologies). The retained mAECs are then plated with EC medium enriched with 10% FBS, 0.1 mg/ ml of heparin (Heparin sodium salt from porcine intestinal mucosa - REF H3393 – Sigma-Aldrich) and 50 µg/ml of ECGF (Endothelial Cell Growth Supplement –ECGS, 354006 –BD) or 5 µg/ ml of bovine brain extract.
12. mAECs should not be used after 7-8 passages.

Isolation of mouse aortic smooth muscle cells (mASMC) (CNIC)

1. Remove the aortas from 5-7 mice (20-25 g body weight and put them in cold PBS (Falcon 50 mL tube).
2. Always work in sterile condition from this step till the end of the extraction (cabinet). Wash aortas with PBS once.
3. Remove the fat around aorta.
4. Incubate the aortas with 0.65 mg/ml Collagenase type 2 (300 U/mg CLS-2 LS004176-Worthington Biochemical Corporation) in DMEM medium (Dulbecco's Modified Eagle's Medium - high glucose D5796, supplemented with 1% Penincilin/Streptomycine) for 20´ at 37 ºC.
5. Remove the adventitia from aorta in culture media. Cut the aortas in rings with a sterile scalpel and put them in DMEM containing 4 mg/ml of Collagenase type 2 at 37 ºC for 40´. Stop reaction by adding DMEM enriched with 10% FBS
6. Pellet the aortic rings (900 rpm 4 min.) and wash 2-3 times with DMEM 10% FBS. Discard the supernatant.
7. Dissolve pellet and culture it in a p60 plate in a small volume of media (2 ml approx.).
8. Incubate for 2-5 days at 37 ºC in DMEM 20% FBS+ 1% Penincilin/Streptomycine +1% L-Glut in a cell culture incubator without movement. Check the cells and carefully change aprox. 2-2.5 ml of culture media until 5th day. (It is important not to remove aortic rings when changing medium).
9. When plate has enough adherent cells, remove media, wash twice with cold PBS, and add 2 mL Trypsin-EDTA solution. Stop trypsin activity by adding 2-3 ml of culture media, recover the detached cells and centrifuge them at 1200 rpm for 5 min.
10. Discard supernatant, re-suspend the pellet and distribute the cells in culture dishes (depending on the obtained pellet).
11. Experiments: Starve cells with DMEM + 1% Penincilin/Streptomycine+1% L-Glut for 48 hours previous to perform the desired experiment.
12. mASMCs should not be used after 7-9 passages.

Isolation of macrophages from mouse bone marrow (CNIC)

Bone marrow-derived macrophages (BMDMs) are obtained by flushing mouse tibiae and femurs with ice-cold PBS and passing the suspension through a cell strainer with a 70 mm cut-off. Cells are seeded in non-treated cell culture plates in 10 ml RPMI 1640 supplemented with 10% L929-cell conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) (L929 cells from American Type Culture Collection, Ref. CCL-1™). Alternatively, recombinant murine M-CSF can be obtained from several customers (i.e Peprotech, Ref. 315-02) and used at 20-100 ng/ml to obtain BMDMs. Cultures are incubated at 37 ºC in 5% CO_2 for 7 days to obtain CD11b+ BMDMs with ~95% purity.

Isolation of mouse peritoneal macrophages (CNIC)

Inject intraperitoneally 3 ml of sterile PBS or saline and give the mice a massage in the belly. Retrieve peritoneal exudate with a 5 ml syringe (20G, yellow) and plate cells in cell culture dishes for 2 h, and wash extensively with PBS or culture medium to eliminate non-attached cells.

Macrophage polarization

BMDMs or peritoneal macrophages are washed and cultured for 24 hours in RPMI 1640 medium supplemented with 10% FCS, and either IFNγ (20 ng/mL) plus LPS (10 ng/mL) (for M1 polarization: "classical activation") or IL-4 (20 ng/mL) (for M2 polarization: "alternative activation").

Murine interferon γ (IFNγ): Ref. 315-05, Peprotech

Murine interleukine 4 (IL-4): Ref. 404-ML, R&D

Lipopolysaccaride (LPS): Ref. L2654, Sigma-Aldrich.

Appendix B – *Supplier Documentation related to Reagents*

As above.

For isolation of mASMC and mAECs

Collagenase type 2: 300 U/mg, Ref. CLS-2 LS004176, Worthington Biochemical Corporation

Gelatin from bovine skin: Ref. G9382, Sigma-Aldrich

Collagen from calf skin: Ref. C8919, Sigma-Aldrich

Purified rat Anti-Mouse CD102 Monoclonal Antibody: Ref. 553326, BD Pharmingen

Dynabeads Sheep anti-Rat IgG: Ref 110.35, Invitrogen

DynaMag-15 Magnet: Life technologies

Heparin sodium salt from porcine intestinal mucosa: REF H3393, Sigma-Aldrich

Endothelial Cell Growth Supplement: Ref. 554006, BD Pharmingen

For isolation of macrophages from mouse bone marrow

L929 cells: American Type Culture Collection, Ref. CCL-1™

Recombinant murine M-CSF: Peprotech, Ref. 315-02

For macrophage polarization

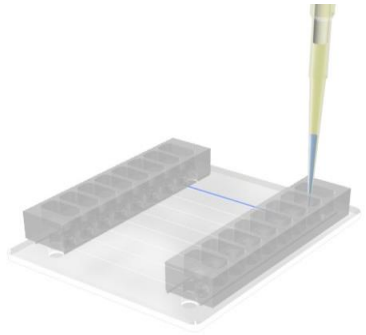
Murine interferon γ (IFNγ): Ref. 315-05, Peprotech

Murine interleukine 4 (IL-4): Ref. 404-ML, R&D

Lipopolysaccaride (LPS): Ref. L2654, Sigma-Aldrich.

For Vena8 Endothelial+ biochip coating and cell seeding

Step 1

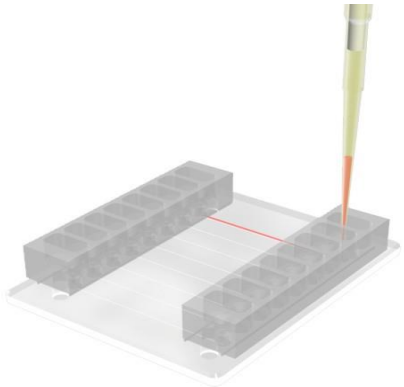


Cellix Vena8 Endothelial+™ biochips are kept under UV for 20 minutes and coated using a standard yellow tip pipette, by dispensing approximately 12 µL of fibronectin into each microchannel. Note the excess of liquid on the entrance and exit ports.

Step 2

The Vena8 Endothelial+™ biochip is then placed in a humidified box, which remains open for 1 - 1.5 hours in the CO_2 incubator. Alternatively, the biochip may be placed at 4 ^{o}C for overnight coating.

Step 3



After the incubation period, add approximately 5 µL of 1.5 x 10^6 per 100 µl of endothelial cells gently into each channel.

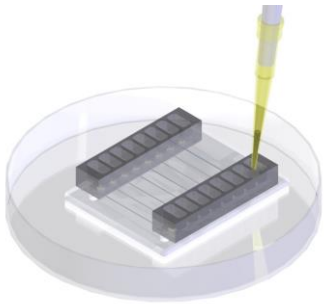
Note: concentration specified is for primary HUVEC.

The biochip is kept in the CO_2 incubator for 15 – 20 minutes. Observe the biochip under microscope and top up all the reservoirs with 40 µL of media. Keep the biochip for 1.5 - 2 hrs in the CO_2 incubator.

For Kima pump preparation and running

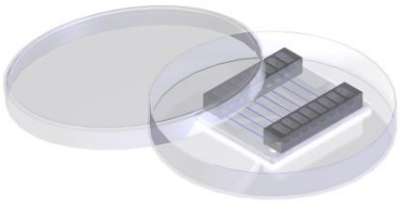
Protocol for Coating and Cell seeding in Vena8 Endothelial+™ Biochips and perfusion using KIMA pump

Step 1



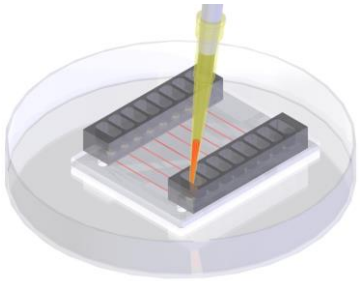
Cellix's Vena8 Endothelial+™ biochips are coated using a standard pipette tip. Dispense ~12 µL of protein (e.g. Fibronectin) into each microchannel. Note the excess of liquid on the entrance and exit ports.

Step 2



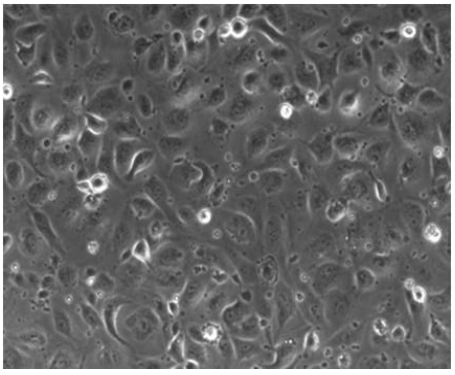
The Vena8 Endothelial+™ biochip is then placed in a humidified sterile petri dish, which should be placed at 4°C for overnight coating.

Step 3



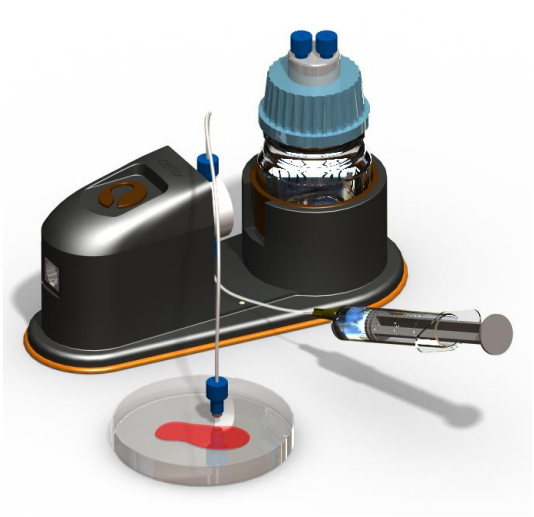
a. The biochip is kept in a sterile petri dish and in the CO_2 incubator for 15 – 20 minutes. Observe the biochip under microscope and top up all the reservoirs with 30µL of media. Keep the biochip for 1.5 - 2 hrs in the CO_2 incubator.

b. After the incubation period, add ~5 µL of 2 x 10^6 per 100 µl (i.e. 20 x 10^6 cells/mL)* of endothelial cells gently into each channel. ***Note:** concentration specified is for primary HUVEC.



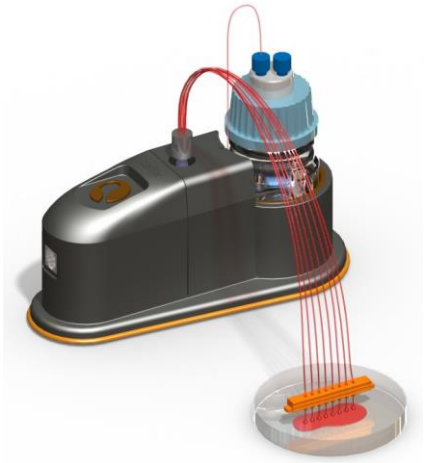
HUVEC primary cells seeded in Vena8 Endothelial+ biochip microchannel: 1.5hrs post-incubation.

Step 4



Wash Kima pump with 70% ethanol and then with sterile distilled H_2O using a 5 mL sterile syringe in the biosafety hood.

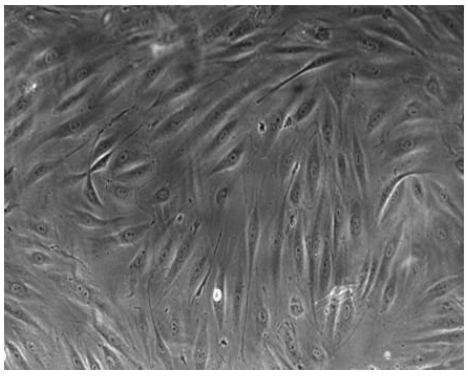
Step 5



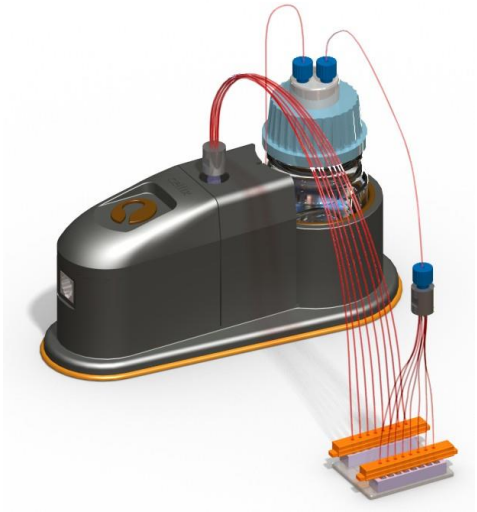
Connect tubing from media bottle to the inlet port of the pump. Connect tubing from outlet port of the pump (8 way cable with pins) to a sterile petri dish. Wash the pump using iKima App with media for 3 minutes.

Step 6

a. Take the biochip from the incubator and place in the biosafety hood. Before connecting to the biochip, start perfusion using iKima App – typically 2 minutes perfusion, followed by 15-20 minutes pause. When media droplets form at the pins, gently connect the 8-way cable to the biochip to avoid air bubbles.



HUVEC primary cells in Vena8 Endothelial+ biochip: 72 hrs post-perfusion with Kima pump



b. Connect the outlet pins to the biochip which is connected to a discard bottle or to the same media bottle for recirculation of the same media. This is done in the biosafety hood. Once connected, transfer Kima platform to a CO_2 incubator.

Note: All tubing and bottles must be autoclaved prior to the experiment.

Appendix C – *Protocols related to Laboratory Evaluation with Artificial Arterial Models*

This section will be developed between month 6 and month 12, after we have defined the targets & specifications.

Appendix D – *Biochip footprint and design drawings*