Development and evaluation of a novel tool for physiologically accurate data generation

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V1	21/09/2012	Preparation of material	A Ahluwalia
V2	24/09/2012	Finalisation	AC de Giacomoni

EXECUTIVE SUMMARY

1. Description of the deliverable content, objectives and purpose

This document gathers documents prepared and used for the training event organised by University of Pisa in Roma.

INLIVETOX WORKSHO: A PRACTICAL TRAINING COURSE ON INTEGRATED FLUIDIC MODELS FOR IN-VITRO TESTING

17-18 September 2012

Organized by

ISTITUTO SUPERIORE DI SANITÀ

Dipartimento Ambiente e connessa Prevenzione Primaria

e

CENTRO INTERDIPARTIMENTALE DI RICERCA "E. PIAGGIO"
UNIVERSITÀ DI PISA

Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, Italy

Building 1, floor C, Room number 74

N° ID: 092C12

The establishment of new in-vitro models requires an evolution of technology in order to allow researchers to implement new and physiologically relevant situations. The word "relevant" refers to the correspondence between in-vitro models and human physiology To this end, a modular fluidics system was developed in InLiveTox to model the response of cells and tissues to ingested nanomaterials. The system is more convenient and ethically less questionable than animal testing as well as more relevant than the in-vitro single cell culture /co-culture models currently being used.

This course has the goal of presenting the InLiveTox concept and fluidic systems to researchers involved in toxicity testing, barrier models and with a strong interest in refining in-vitro test systems as a means of reducing animal tests. These attractive and low cost devices will allow researchers to surpass the problems related to simulation of a physiological tissue in-vitro, in order to obtain a relevant model of an organ or multi organ system.

Language: the official language of the Course will be English

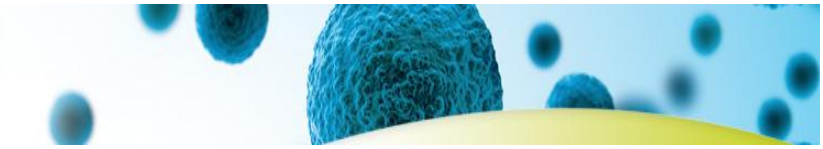
SCIENTIFIC PROGRAM

Monday, September 17 (theoretical part)

12.00	Participants Registration and snacks
14.30	Introductory Remarks - *Arti Ahluwalia*
14.50	Alternative testing and CELLTOX mission - *Isabella De Angelis*
15.10	Overview of research on in-vitro alternatives to animal testing - *Candida Nastrucci*
15.30	The business of alternatives - *Malcolm Wilkinson*
15.50	Overview of nanotoxicology - *Flavia Barone*
16.10	Theoretical description of fluidics and scaling - *Arti Ahluwalia*
16.30	Introduction to the bioreactors Logistics for laboratory - *Tommaso Sbrana*
16.50	InLiveTox system (cells test and monitor) - *Sher Ahmed*
17.10	General discussion
17.30	End of the day
19.30	Optional conference dinner

Tuesday, September 18 (practical part)

8.30	practical demonstration of fluidic system set up - *Tommaso Sbrana*
10.30	*Coffee break*
11.00	Cell in bioreactor (Lab-Module II) - *Sher Ahmed*
12.30	*Lunch*
14.00	practical demonstration of membrane bioreactor set up-*Tommaso Sbrana*
15.30	*Coffee Break*
16.00	TEER measurements and cells assays (Lab - Module III, IV) *Sher Ahmed*
17.30	participants evaluation test



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A PRACTICAL TRAINING COURSE ON INTEGRATED FLUIDIC MODELS FOR IN-VITRO TESTING

17 – 18 September, 2012, ISS Rome (ITA)

In this document the reader will find a brief description of the InLiveTox project and the bioreactors that have been used to establish the in-vitro models. This section has the goal to provide the information cited during the presentation in the workshop theoretical part.

Overview of in-vitro tests

There has been a huge drive to refine and develop new in-vitro methods that could be used to reduce animal testing. Making more advanced models which are closer to the *in vivo* situation is of utmost importance in order to validate tests and to commit to the 3Rs approach. New EU directives state that animals should only be used as a last resort. New in-vitro alternatives are thus aimed at developing models that are more representative of the physiologic environment than classical cell monolayers. In particular a new technology has been developed in order to substitute static cultures devices: bioreactors. These devices have several shapes and dimensions on the bases of their task and their function. Bioreactors allow cell culture in sterile conditions in a dynamic environment with the presence of time dependent concentrations of nutrients or drugs and velocity gradients.

Dynamic culture conditions systems have several advantages, in comparison to static culture systems. Briefly they are summarized below:

- Possibility to impose pressure gradient or shear stress in order to stimulate cells.
- Possibility of nutrient exchange during the experiments as in the physiological environment.
- Higher oxygenation rates compared with static cultures, due to the exchange of media.
- Possibility to stimulate cross talk between different cell types.

Introduction: the InLiveToxproject

The goal of the FP7 InLiveTox project was to develop an improved in-vitro model to study the impact of Nanoparticle (NP) exposure on the body. In Europe and in the USA, governments, non-governmental organisations, and others have expressed concern that, with the field of nanotechnology and the number of

consumer products incorporating nanomaterials increasing dramatically, in many cases, the safety of these materials has not been demonstrated and there are still a large number of unanswered questions regarding modes of entry into the body and into cells. The challenge for health (and environmental) protection is to ensure that as nanomaterials are developed and use, any unintended consequences of exposures to humans are prevented or minimised. In order to understand behaviour and responses of biological systems to nanomaterials, and therefore to manage subsequent risks, it is essential to investigate the hazard (toxicology) of the large number of engineered NP in different formulations and at different points in their life cycle (from production to disposal), in relation to different routes of exposure and different target organs and tissues. The number of experiments therefore required to address such issues is enormous and so it is essential to develop rapid and reliable non-animal models to assess NP hazard

The model developed by the InLiveTox consortium is based on a modular fluidics-based multi compartmental cell culture system (abbreviated ILT in this document) aimed at recapitulating NP absorption in the intestinal wall, distribution through the vascular network and metabolism in the liver. Different tissue models based on human cells are cultured in the compartments of the ILT and are connected via the flow of culture medium through the fluidic system (Figure 1). Although the scheme described may seem simplistic, it has already been shown that even 2 cell connected culture systems behave in a more physiologically relevant manner than static monocultures [1,2].

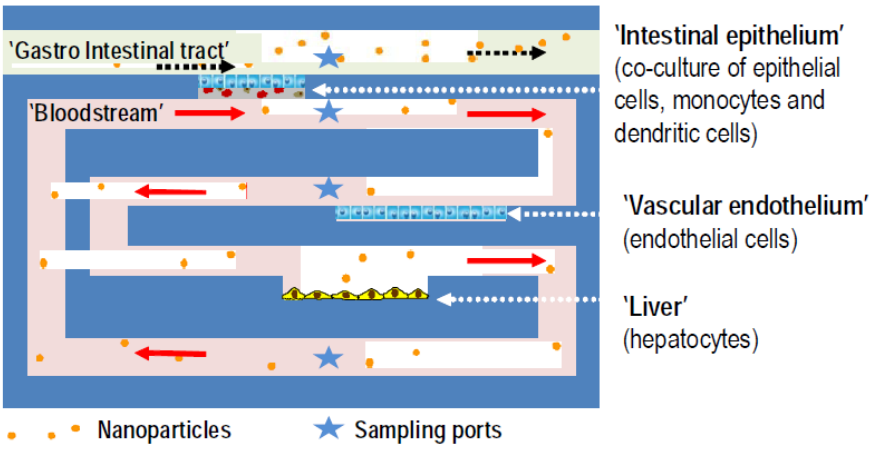


Figure 1: Basic InLiveTox (ILT) model

ILT system overview

The ILT system consists of a small number of cell culture compartments linked by a fluidic system (Figure 2). The compartments are designed for the culture of models of the intestinal epithelium and other cells such as hepatocytes and endothelial cells. In addition to the fluidic device and external control and analytic

instruments, a number of liquid containers are also associated with the ILT system. These bottles and/or chambers function as reservoirs of the liquids to be used (e. g. cell culture media) as waste containers, as mixing chambers and as gas exchangers. The ILT system is modular so that compartments and fluidic circuits can be combined in different ways in order to simulate different biological models. The ILT circuit is composed of two different types of chambers: ILT0 bioreactors mimic organs such as the liver, and the endothelium, ILT2 houses a membrane and permits the simulation of the intestinal epithelium.

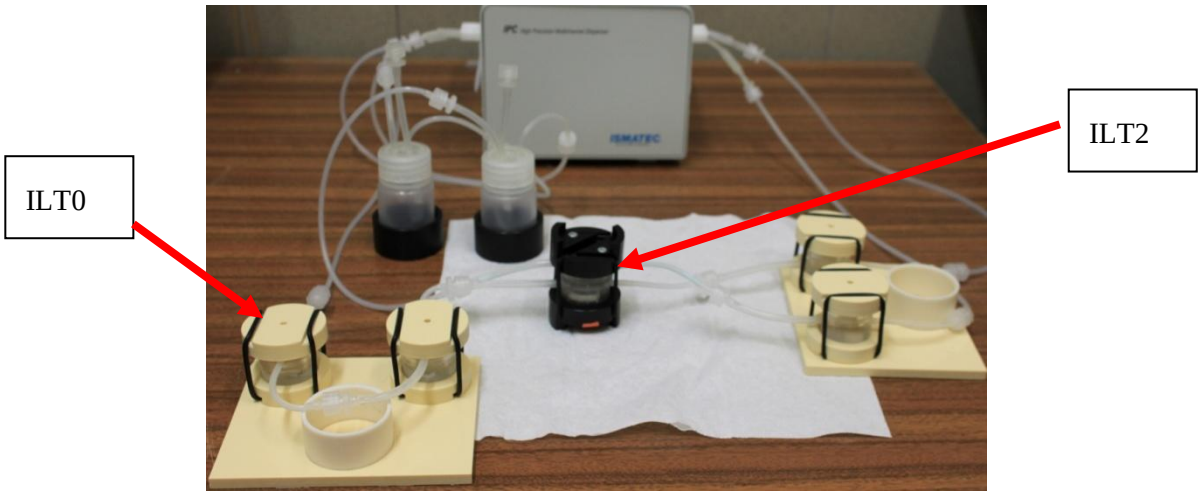


Figure 2: The basic set- up: ILT2 is joined with 2 ILT0s. Each bioreactor chamber houses a different cell type. Two mixing chambers are required because of the double flow over the membrane.

In next paragraphs a brief description of devices is provided. Three bioreactors (ILT0, ILT2 and MCmB3) are presented. ILT0 is the original bioreactor chamber developed at the University of Pisa, now commercialized as the Quasi-Vivo chamber. MCmB3 is simple membrane version of ILT0. ILT2 was developed in the InLiveTox project by CSEM (Centre Suisse d'Electronique et de Microtechnique) in Switerland and University of Pisa.

ILT0 description

Bioreactor ILT0 has been described in various publications [3]. It is composed of two parts, an upper and a bottom one, realized in PDMS (polydimethylysiloxane). PDMS is biocompatible, transparent and self sealing. Its main features are:

- Absence of air bubbles
- Low shear stress
- High flow rates

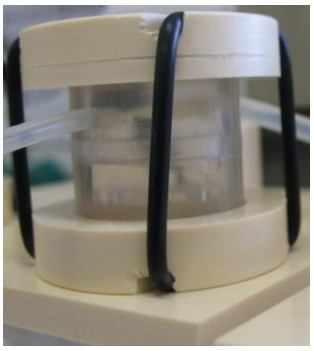
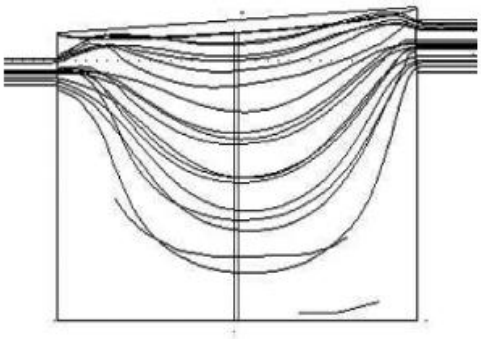
A  B 

Figure 3. A: 3D view of ILT0. B: Laminar flow evaluation inside the bioreactor. The study was performed using a computational fluid dynamic model.

As shown in figure 3 a clamp system is required in order to prevent leakage between the upper and bottom chambers. The commercial system does not require clamps because of the twist opening mechanism.

ILT2 description

ILT2 represents an important evolution of ILT0. The main advantages of ILT2 compared to ILT0 are the possibility to impose a double flow of media above and below a semi-permeable microfabricated silicon nitride membrane, suitable to mimic physiological barriers and to enable the placement of electrodes inside the bioreactor to measure Trans Epithelial Electrical Resistance (TEER) in real time continuously. This bioreactor is composed of an upper and a bottom chamber , electrodes and a holder containing the membrane interposed between the previous two parts.

Figure 4: ILT2 showing the clamps, tubing and electrical contacts.

MCmB3 (modular chamber membrane bioreactor) description

MCmB3 permits the establishment of air-liquid interface or liquid-liquid interface models to study physiological barriers using commercial membranes used in transwells. It is simpler in design than the ILT2 and cannot measure TEER.

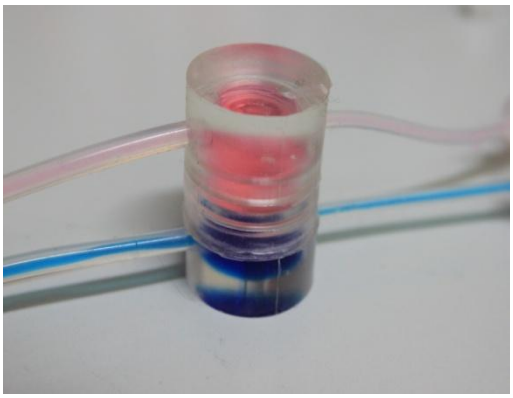


Figure 5A: insert for commercial membranes. B: MCmB3 with 2 different solutions separated by a membrane. It is possible to have different interfaces such as liquid- liquid or liquid-air.

Practical Course

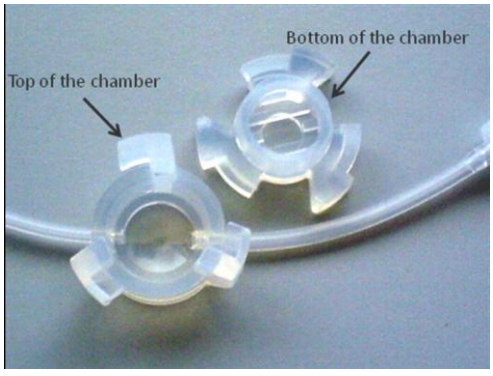
In this practical course you will learn how to i) set up a one and two chamber flow experiment using the ILT0 or Quasi-Vivo ii) assemble the ILT2 and perform a TEER measurement under double flow conditions iii) assemble and set up the MCmB. Finally, we will describe the InLiveTox system and show how it can be used to build a fluidic circuit for simulating NP absorption across the intestinal barrier through to the vascular circulation and liver.

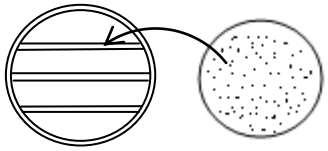
i) ILT0

ILT0 or QV chambers are made of PDMS poly-dimethyl-siloxane, a biocompatible silicone. Gas-plasma sterilization is used to sterilise the chambers as it is the most efficient method which maintains the unique properties of silicone (flexibility and permeability). The sterile packs should be opened under a hood.

a) cells on coverslips

Flow experiments require cell confluencey and a good degree of adhesion. Therefore cells should be seeded at least 24 hours before the experiment on pre-treated 12-13 mm glass or plastic coverslips (typically using a 24-well plate). The coverslips can be pre-treated by coating with collagen I (from rat tail) or gelatin as required. Take the coverslips with the cells from the wells and put each one on the bottom of a chamber, with the cells uppermost (figure 1). Put one drop of growth medium onto the cover slip to prevent drying out of the surface.





13 mm coverslip with cells

Figure 6. Putting the coverslip onto the bottom of the chamber

b) opening and closing the chambers

To open the chamber grip the bottom of the chamber between three fingers, one on each of the lugs and twist the top of the chamber in an anti-clockwise direction. Lift off the top of the chamber. To close the chamber push the top onto the bottom then grip the bottom of the chamber between three fingers, one on

each of the lugs and twist the top of the chamber in a clockwise direction, locking the chamber when the external lugs are fully engaged.

c) reservoir or mixing chamber

Fill the reservoir bottle with the medium of your choice; approximately 4 ml per chamber plus some extra medium. For example, for 2 chambers we suggest adding between 10-20 ml of medium. The volume of medium used depends on the length of the culture period and can be optimised for the specific cell type. Carefully screw on the top of the reservoir bottle. If highly proliferative cells are used, it is advisable to use medium with no serum added, to prevent excessive cell proliferation.

c) setting up the tubing and pump.

The main thing to remember is that the smaller tube 1/16" ID tube is the inlet and the big 3/32" ID tube is the outlet in all chambers. Following the system round in a loop; the outlet tube of the reservoir is connected to 1/16" ID extension tube (22 cm in length). This is connected to the pump manifold tubing and inserted into the pump rollers. The pump output which is in turn connected to the 3/32" ID extension tube (22 cm in length). This is connected to the chambers which are then connected to the reservoir inlet. Figure 7 shows three chambers connected in series, however, the number of chambers employed may be adjusted for the particular experiment. Once the system is connected in this way it is recommended not to disconnect the tubes or chambers as this will increase the risk of infection. The system is now closed and the experiment may begin; the system should be placed inside a CO_2 incubator with the pump.

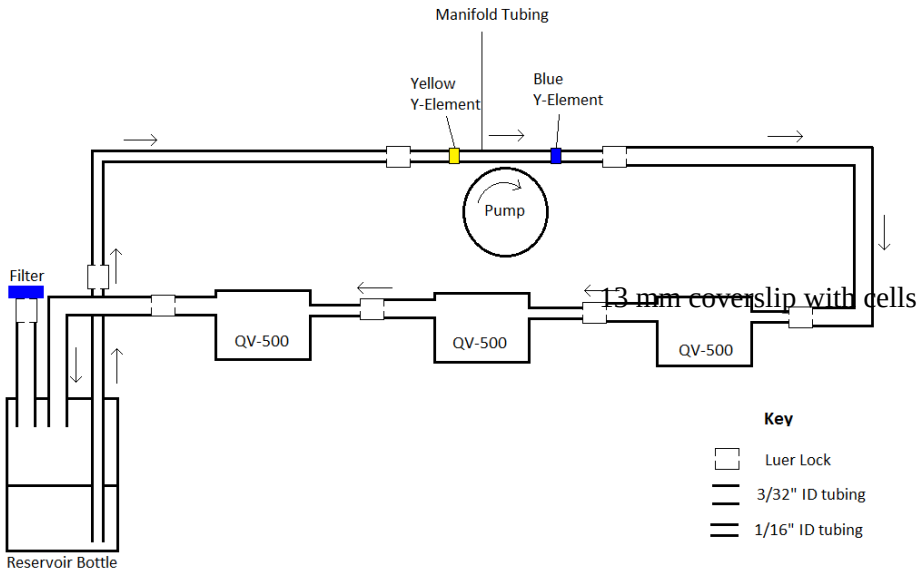


Figure 7: Schematic of the system

We will discuss aspects such as sampling, pumps and flow rates as they depend on the type of experiment and cells being used.

ii) ILT2

ILT2 was developed for simulating biological barriers. Its main components are:

a)The ILT2 chamber Silicon Nitride Membrane Chips

The ultrathin (500nm) porous silicon nitride membranes were developed at CSEM (Centre suisse d'électronique et de microtechnique) in Switerland and the key features are low membrane thickness and the presence of 4 platinum electrodes for enabling TEER measurement in the ILT 2 bioreactor system. Each membrane consists of 23 porous pads which are 1mm x 1mm. The polymer membranes used in transwell inserts are not suitable for the study of nanoparticle passage because they get trapped in the pores.

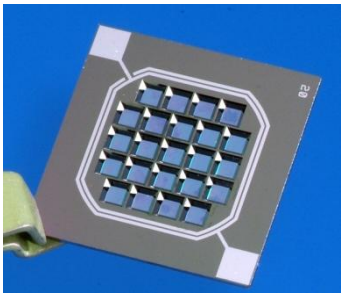


Figure 8. Shows a silicon nitride membrane chip with platinum electrodes

b) ILT2 clamp and electrical contacts

A clamp (Figure 9) was also designed to allow the insertion of gold contacts old contacts to touch the platinum electrodes for the TEER measurements.

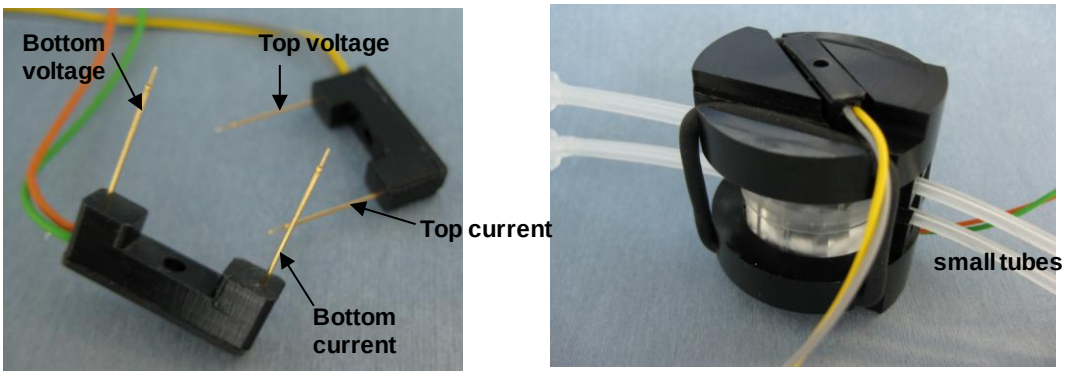


Figure 9. Left image shows the electrode contacts and which are for current and which are for voltage. The right image shows the bioreactor system set up with clamps-

c) Membrane holder

A membrane holder system is designed to hold the silicon nitride microfabricated membrane and permit handling without damaging the delicate chip. The holder also enables accurate placement of contacts on the electrodes as well as cell seeding and culture in petri dishes or plates prior to insertion in the bioreactor. This is useful because barrier cells usually require a long culture period before forming a differentiated epitheial monolayer.

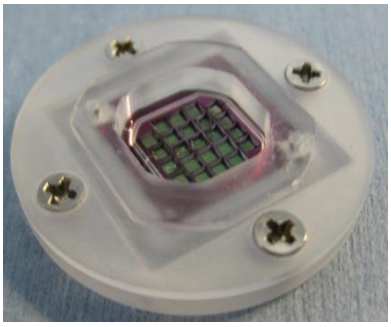


Figure 10:This is the holder for the silicon nitride chips.

ILT2 Assembly

After the holder with membrane has been inserted into the bioreactor we will show you how to assemble and fill the 2-flow circuit and take samples during an experiment.

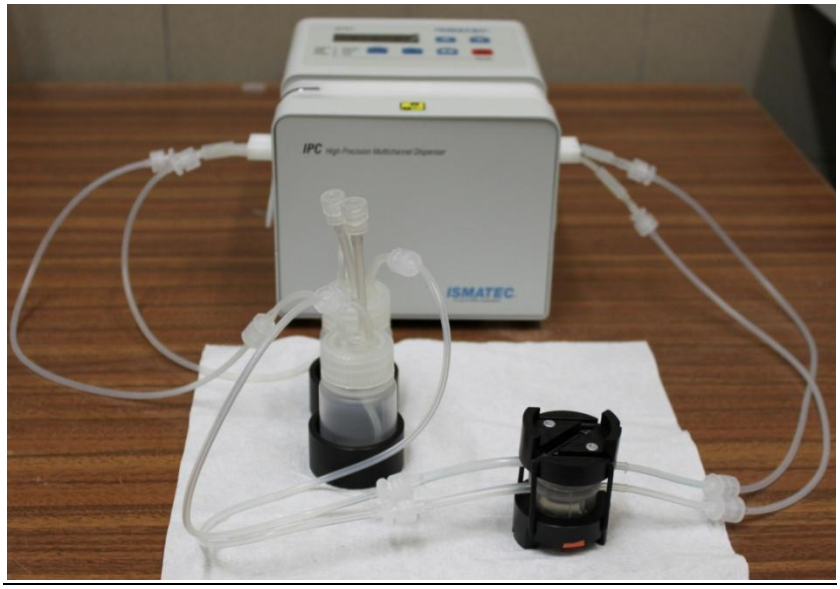


Figure 11:Complete ILT2 two-flow circuit for simulating biological barriers

Cell Model

The chosen cell line for use in the model is the caco-2 (C2BbE1) cell line. This is a polarised colon cell line and the cells form functional tight junction after 21 days in culture. They typically proliferate for 10 day and then differentiate into entrocytic type for approximately another 11 days. By the end of the 21 days these

cells form good tight junctions and exhibit good TEER values. TEER values should be in the range of 300-600Ω.cm^2

Cell Seeding on Membranes

When using Caco-2 cells. Approximately 100,000 cells should be added per membrane chip. This depends on the cell type used. For using the ILT2 bioreactor the ILT2 polycarbonate holder should be used. Cells are grown using this holder for 21 days before being transferred into the PDMS bioreactor.

- The polycarbonate holder should be placed in a 6 well plate
- About 2ml of media should added to the bottom of the plate. The holder should *not* be covered in media at this point. Care should be taken so that a bubble doesn't form at the bottom side of the membrane
- 100,000 cell should be diluted in 1ml of cell culture media and added to the top side of the membrane
- The cells should be allowed to attach for approximately 1.5-2 hours
- The ILT2 holder can then be gently submerged in cell culture media and allowed to culture normally until transfer into the bioreactor.

How to take samples

During experiments it could be useful take samples in order to evaluate concentration of NPs or metabloites at different times. Three way valves will be used to permit this operation. One valve will be positioned in the upper circuit, after ILT2. The other one will be placed in the bottom circuit. Maintaining the pump on, the operator will turn the valve, in order to open the communication between circuit and outside environment. Gilson will be used to take liquid samples, waiting that the liquid will flow out from the valve, according to pump pressure. Don't try to take liquid samples before the right time, otherwise air could be flow inside Gilson tip. At the end, close the valve in the starting position.. Repeat the same operation with the other valve. It is important to make this operation as fast as possible in order to avoid a liquid loss and a pressure drop inside of the circuit.

How to monitor cells using TEER Measurements

Transepithelial electrical resistance measurements are a measure of the integrity of a monolayer of cells. These measurements are only valid with certain cells types. These cell types are usually epithelial cells which exhibit tight junctions to give a tight monolayer of cells that restricts the flow of ions from the either side of the cell layer. When there is cell damage a reduction in TEER value is seen. Traditionally cells are seeded on Transwell polymer inserts and TEER measurements are taken using STX2 chopstick electrodes and an EVOM multimeter (Figure 11) . In ILT2 a 4 point measurement system is used where by two electrodes inject a known current into the system and the other two electrodes measure the resulting voltage. This is then converted into a resistance value. To obtain the TEER value from this resistance measurement

the background resistance (resistance without cells) must be subtracted and then this number is multiplied by the surface area of the porous support. TEER is given as '$\Omega.cm^2$'

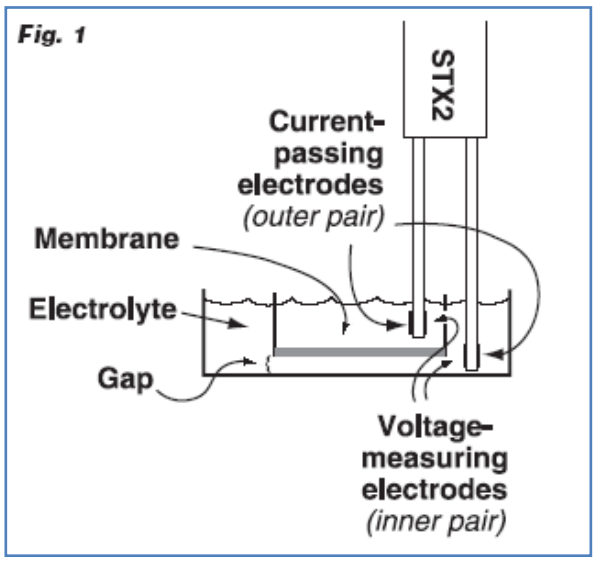


Figure 11. scheme of TEER measurement electrodes (STX1) in presence of an permeable membrane where cells are seeded.

iii) MCmB3

This chamber is simpler than the ILT2. However it can only be used for investigating the passage of soluble materials like drugs and chemicals. The insert is a simple PDMS device which is holds 24 mm filters or membranes which can be purchased from Fischer. The insert is placed between the top and bottom chamber of a two flow ILT0. The circuit connection is similar to that of ILT2. We will show examples of dye passage through the system.

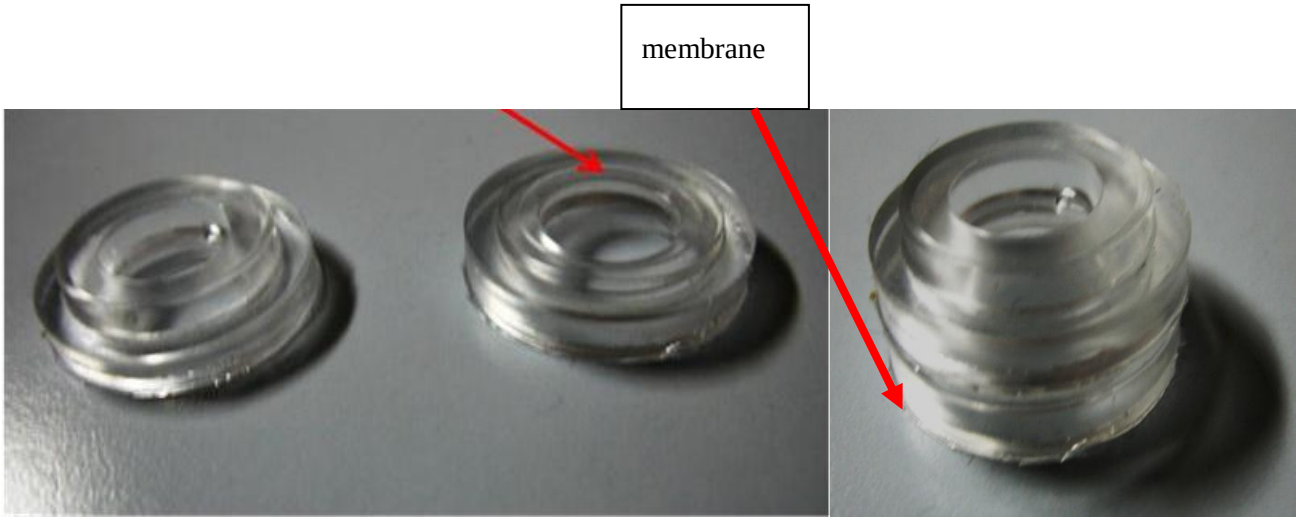


Figure 12: MCmB3 inserts

Appendix: Assays and further details on cell culture

Alamar Blue assessment

- After the incubation time, remove the supernatant from the cell cultures (store for other assays or discard)

- Wash the plate once with warm PBS to remove remaining medium, cell debris or particles

Add 100 µl of fresh medium containing 10 % Alamar Blue reagent per well of a 96-well-plate; for larger cell culture chambers, adjust that volume accordingly.

- Incubate at 37ºC (see note below for times) and read plate on a fluorescent plate reader (excitation: 540-570 nm; emission: 580-610 nm). Viability can be expressed directly in fluorescent units, or as % of the untreated control using the following formula:

$$Viability\ (\%) = \left(\frac{Fluorescence(untreated) - Fluorescence(test)}{Fluorescence(untreated) - Fluorescence(Triton - X)}\right) x100$$

- Incubation times for Alamar Blue Assay (static conditions):

    - Caco: 30 min and 120 min

Protocol for Seeding Cells onto glass coverslips

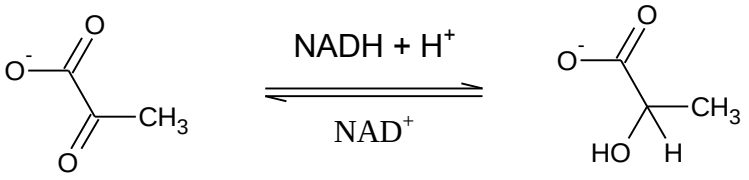
- 13mm coverslips can be sterilised in 100% Ethanol.
- Slides can be pre-treated with collagen I beforehand. This is not required for Caco-2 cells
- Cells should be seeded 24 hours before the experiment using 24 well plates
- Normal cell culture procedures should be used for trypsinisation and counting of cells
- $1x10^5$ Caco-2 cells should be added to each cover slip and left to proliferate at 37 degree centigrade in 5% CO_2

LDH Assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, which is released from cells into media (*in vitro*) or biological fluid (*in vivo*) when the cellular membrane is damaged.

Within the cell, LDH catalyses the NADH-dependent reduction of pyruvate to lactate (Figure 1). This reduction is exploited in the LDH assay, where membrane damage of cultured cells is assessed by the amount of LDH leaked into the cell culture medium.

Figure A1: Pyruvate conversion by LDH:



Pyruvate Lactate

The assay measures the reaction of pyruvate with 2,4-dinitrophenylhydrazine, forming a reddish-brown product which can be quantified photometrically (Figure 2). The more LDH is released from dying cells, the more pyruvate in the reaction mix is converted to lactate, and the less pyruvate will be available for reaction with 2,4-dinitrophenylhydrazine.

Typically, the LDH assay uses a range of concentrations of the agent to be examined for cytotoxicity, untreated cells (negative control) and cells treated with 0.1% Triton X-100 in medium (positive control). Triton X-100 is a detergent which destroys the cell membranes and thereby causes 100% cell death and leakage of LDH into the cell culture medium.

Whenever testing a new particle, some additional wells must be included, in which cells are incubated with both Triton X-100 and particles, to check for LDH enzyme adsorption onto the particles (reduced LDH activity).

Particles must also be tested in medium, but without any cells, to account for any direct interference of the particles with the assay, and interference of the particles with the measurement (i.e. light absorption).

LDH release can be measured quite easily using kits. (Example: TOX7 kit from Sigma or Cytotoxicity Detection Kit, No 11 644 793 001 from Roche)