EXPERIMENTAL REPORT

ADHESION ASSAY STUDY (SP160400)

Establishment and optimization of an adhesion assay with primary human coronary artery endothelial cells (HCAEC)

Program Name	Systems Biology Development, Application and Verification
Project Name	Cellular Systems Biology
Work Package Name	CVD Functional Assays
Experiment Leader	Carine Poussin
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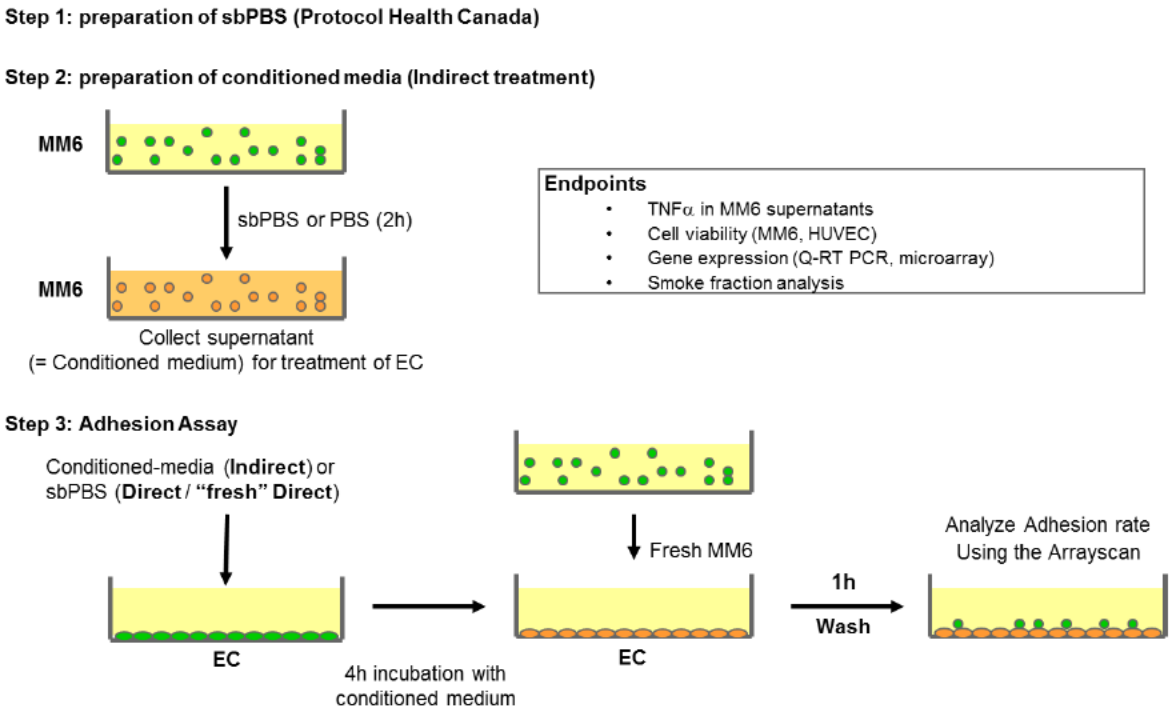
Date:	05/12/2013

Background

The adhesion of leukocytes to the endothelium constitutes a key step in the initiation of atherosclerosis in pathophysiological conditions. To mimic the process of adhesion in vitro, an adhesion assay has been developed previously using human umbilical vein endothelial cells (HUVECs) and Mono Mac-6 (MM6) cells (Report_Adhesion_Assay_Phase_I.docx). The objective of this study was to establish and optimize an adhesion assay using human coronary artery endothelial cells (HCAEC), a more disease-relevant endothelial cell type. The newly established and optimized adhesion assay with HCAECs has been used to test the effect of 3R4F sbPBS (Health Canada), and will be leveraged to assess the effect of MRTPs in the next experimental phase. The scientific background supporting the project is described in detail in the work package charter: "BSR_S2D_PLN_2012_Work Package Charter_Cellular_Systems_CVD" (https://disco.app.pmi/disco/drl/objectId/0901d4ec803689d1), and the business case "BC_Validation_CVD_Assay" (https://disco.app.pmi/disco/drl/objectId/0901d4ec802c7cf5).

Principles of the adhesion assay



Results

Data

The raw data for each experiment are available in folders titled "ExpXXX" located in: \\chp708.lsdc.pmi pmrdcellres\studies\160400_adhesion_assay_establishment\2013

The establishment of HCAECs culture

Effect of collagen-A coating on HCAECs culture

HCAECs were initially grown in the MV1 medium did not grow well in this medium. Therefore, the MV1 cell culture medium was changed for the MV2 medium (supplemented with growth factors). The cells grew normally in the MV2 medium with a population doubling time (PDT) in average of 30 hours (passages 3 and 4), and increasing up to 120 hours for passage 6 in non-coated flasks. Growing HCAECs in collagen A-coated flasks greatly reduced the PDT for higher passages (50% reduction for passage 6) (Figure 1).

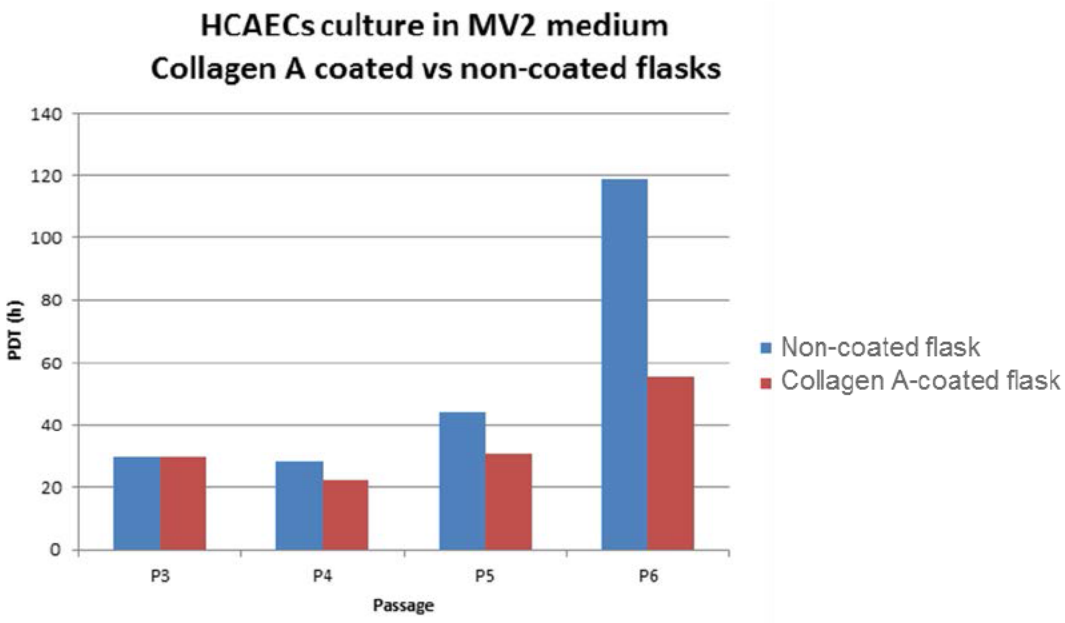


Figure 1: Population doubling time of HCAECs over cell passages when cells were grown in collagen A-coated and non-coated flasks (PDT: populstion doubling time).

A detailed protocol for HCAECs culture is provided in the "Experimental protocols" section of the report.

The iCELLigence system was used to optimize HCAEC culture and experimental conditions for the adhesion assay. The iCELLigence system is an impedance-based real-time cell analysis system which allows for label-free, dynamic monitoring of cellular events. The system measures electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture wells of E-Plates. The impedance expressed in arbitrary cell index (CI) units corresponds to a measure of resistance influenced by cell adhesion, spreading and morphology which change upon cell culture/treatment conditions, cell viability and number. For example, when cells are seeded in cell culture medium, the CI rapidly increases in the first 2-3 hours which has been described to correspond to the adhesion of cells to the substrate. Then, the increase of the CI becomes less important generally following a linear curve that reflects the cell proliferation phase. After a time period that can vary with cell seeding density, type and culture medium, the CI reaches a plateau associated with slowed down proliferation of cells. If cells are kept longer in culture after reaching confluency, the CI decreases suggesting morphological change of cells that could be explained by cell death and/or detachment in this context.

The results obtained with the iCELLigence system used to monitor HCAEC proliferation in the presence or absence of substrate confirmed the importance of collagen A for an optimal growth of HCAECs. Indeed, HCAECs growth rapidly slowed down and even stopped after the seeding of cells in non-coated compared to collagen-coated flasks (Figure 2).

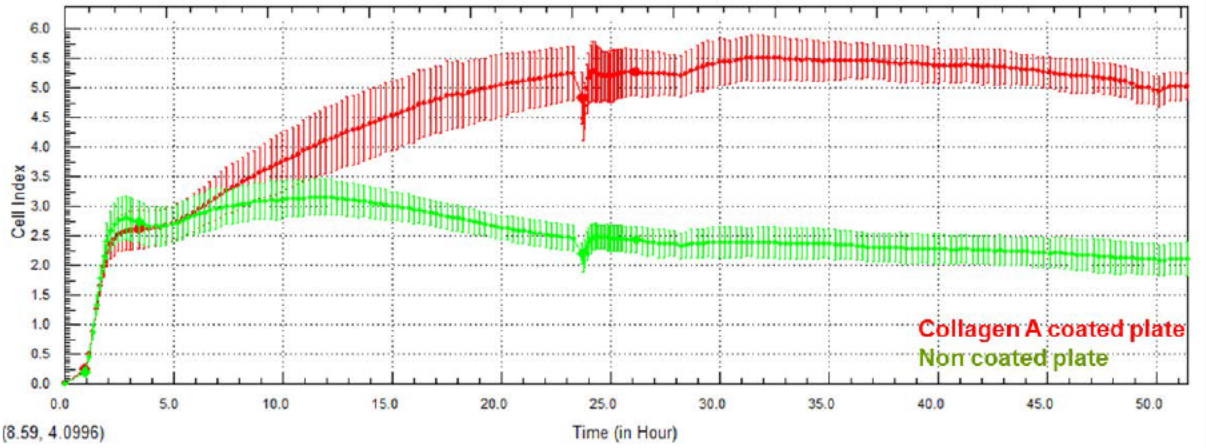


Figure 2: Effect of substrate (collagen A-coated versus non-coated E-plates) on HCAEC cell index over time (Mean ± SD, N=1; n=4) (Exp041)

Effect of seeding densities on HCAECs culture

The growth of HCAECs seeded with different densities was monitored with the iCELLigence system for more than 50 hours. The results showed that HCAECs adhere to the substrate within 2 hours irrespective of the cell density (10 or 20k cells/well). Then, the CI curves indicated that HCAECs proliferate at higher rates for a cell density of 20k compared to 10k, however reaching a plateau at 25-30 hours while HCAECs seeded at 10k cells/well continued to proliferate up to 50 hours (Figure 3).

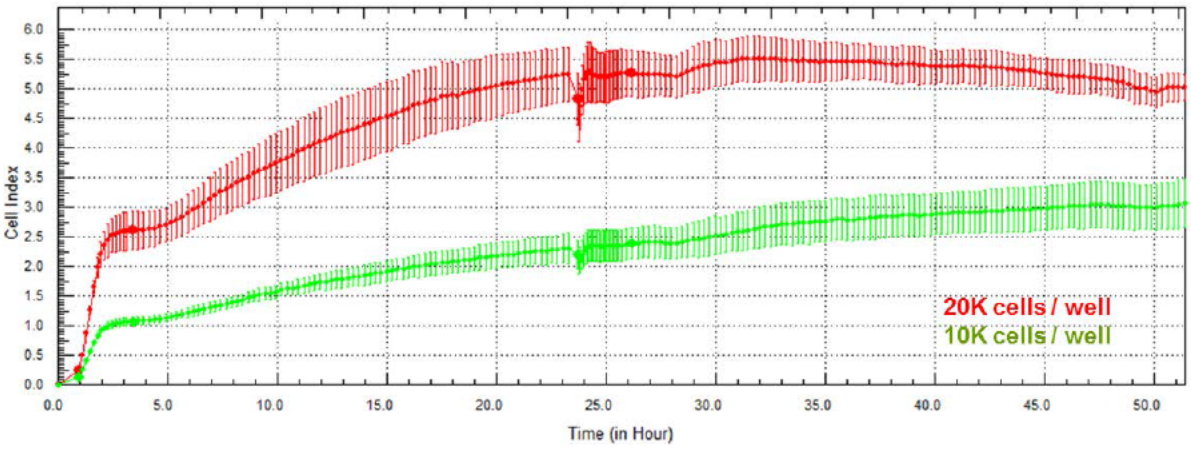


Figure 3: Effect of seeding density on HCAEC cell index over time (Mean ± SD, N=1; n=4). (Exp041)

Effect of full versus partial medium change on HCAECs

Importantly, an experiment using the iCELLigence system assessing the impact of medium change on HCAECs revealed that these cells were highly sensitive to medium change when 100% of medium was removed and fresh one was added. Indeed, CI dropped by 60% indicating a drastic change of cell morphology following the medium change. When only 70% of the volume of medium was changed no significant effect was observed on the CI. These observations suggest that HCAECs are very sensitive to air exposure during medium change (Figure 4).

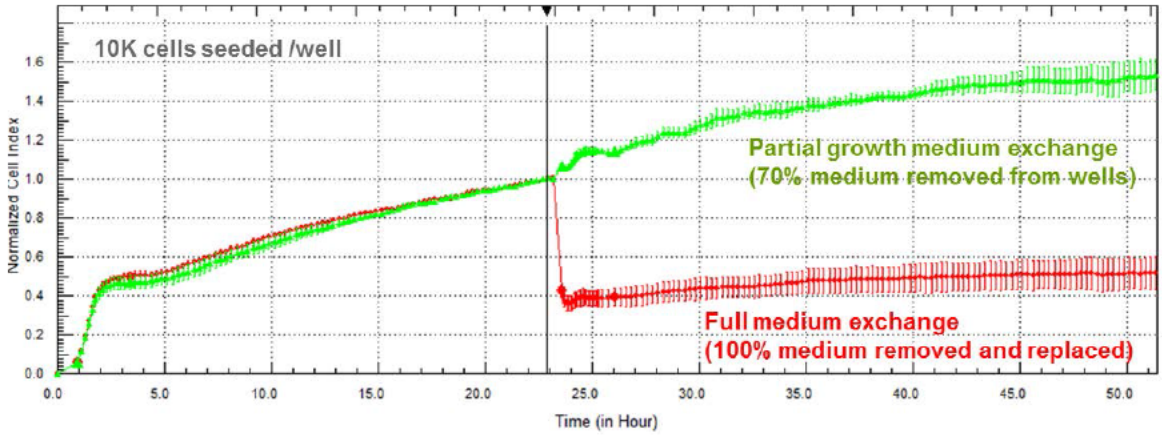


Figure 4: Effect of full versus partial medium change on HCAEC cell index over time (Mean ± SD, N=1; n=4). (Exp041)

Effect of starvation medium on HCAEC

As HCAECs are grown in starvation medium 24-hour prior the adhesion assay, it was important to measure the impact of the starvation medium on those cells over time using the iCELLigence system. The results showed a rapid and transient drop of the CI just after the medium change. Then, CI values which remained 15% lower than the ones measured for HCAECs culture in normal growth medium increased regularly over time, indicating that HCAECs continued to proliferate in starvation medium. The CI reached a plateau after ~13 hours of starvation suggesting that HCAECs stopped proliferating (cells might be synchronized) while

cells in normal growth medium continued to grow. In conclusion, HCAECs can be grown in starvation medium before and during the time period of the adhesion assay (Figure 5).

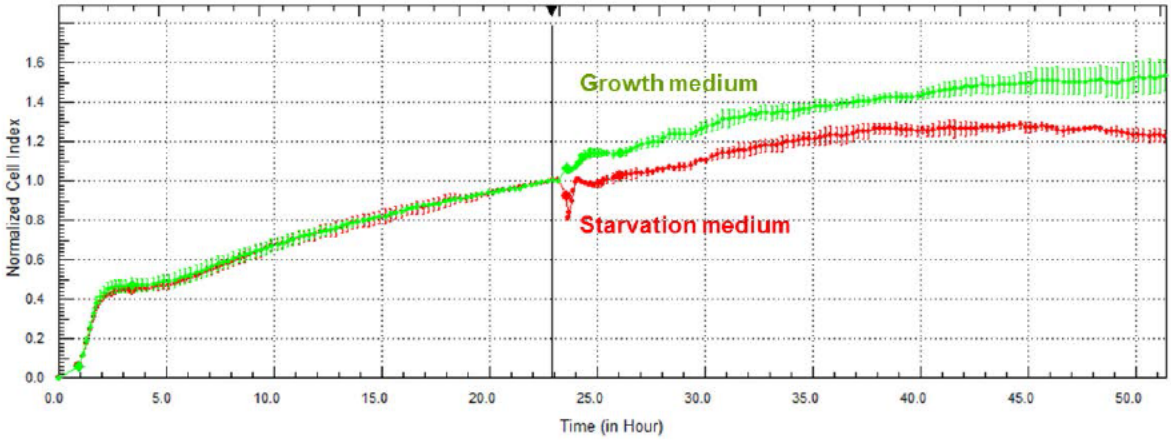


Figure 5: Effect of starvation medium on HCAEC cell index over time (Mean ± SD, N=1; n=4) (Exp041).

Effect of PBS in starvation medium on HCAECs

Depending on the concentrations of sbPBS used to treat cells, the volume of PBS per volume of starvation medium increases with higher concentrations. Therefore, it was important to investigate the effect of PBS (varying the percentage) on HCAEC cell index over time. The results show that no significant effect was observed on HCAEC cell index up to 20% of PBS in the starvation medium, corresponding to the maximum volume of PBS tested (Figure 6).

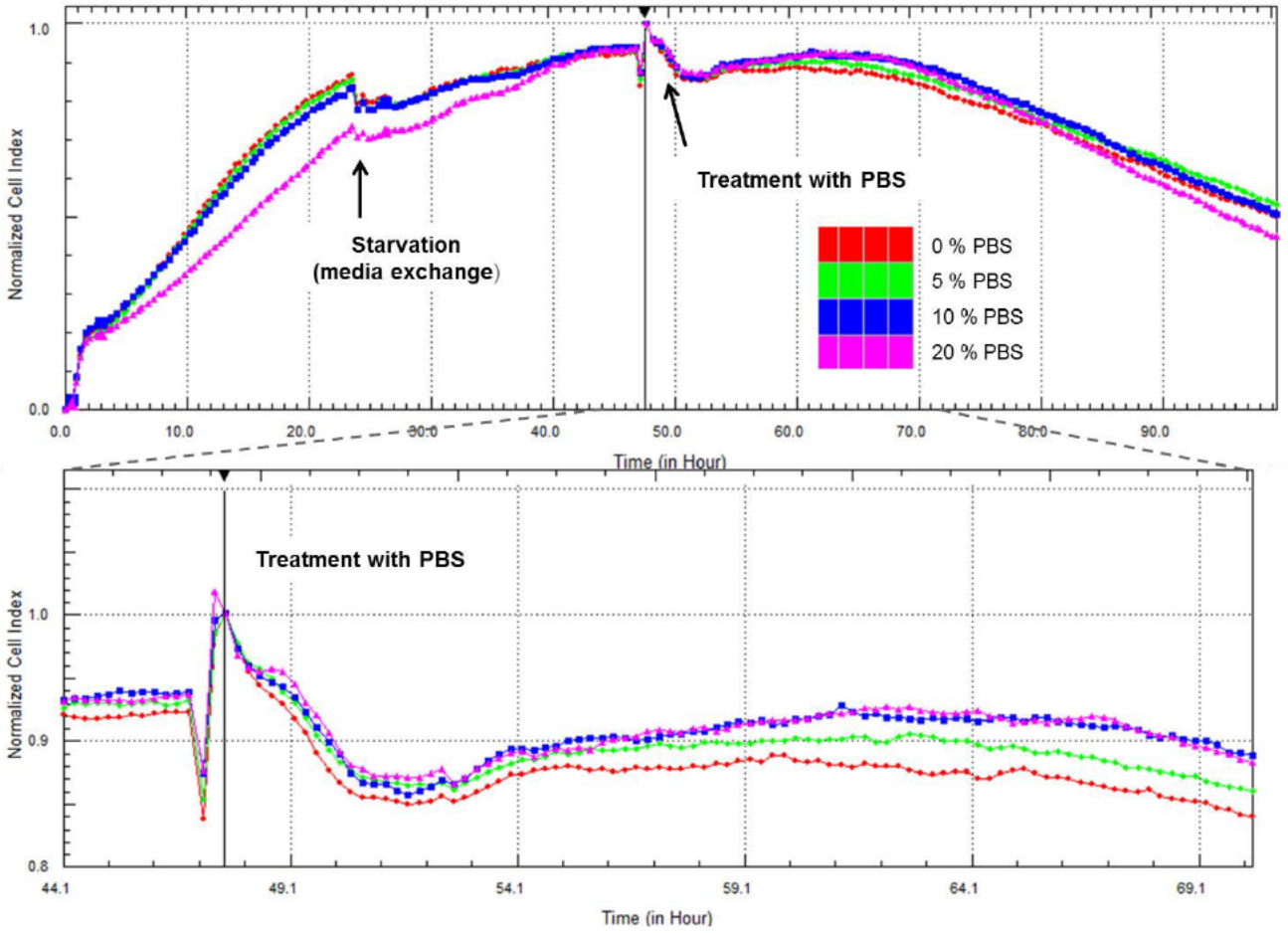


Figure 6: Effect of different percentages of PBS in starvation medium on HCAEC cell index over time (seeding density 15K/well) (Mean ± SD, N=1; n=4) (Exp042).

Adhesion assay set up and optimization with HCAECs

Establishment of the adhesion assay with HCAECs and switch from 48- to 96-well plate format

The adhesion assay has been previously established with HUVECs in a 48-well format. However, the switch from 48- to 96-well format would save precious HCAECs and increase the number of conditions that can be tested in parallel. Different cell densities, starvation duration and effect were tested with HCAECs for the adhesion assay. The adhesion assay was performed using TNF-alpha (10 ng/mL) as positive control and medium only (MM6 starvation medium with 0.5% FCS or HCAEC growth medium with 2% FCS) as

negative control. The adhesion assay was conducted in parallel in a 48-well plate with previously established conditions for comparison.

The experiment aimed at testing different factors that might impact the adhesion assay using HCAECs:

- Cell densities: 15, 20, 25, 30K HCAECs
- Starvation period: 1, 16, 24 hours
- Medium: HCAEC starvation medium (0.1% FCS), HCAEC growth medium (2% FCS)

Briefly, 24 hour after seeding HCAECs, the cell culture medium was changed with fresh medium or with starvation medium. After 24 hour, the adhesion assay was performed.

The results show that TNF-alpha induces the adhesion of MM6 to HCAECs. The difference of the percentage of adherent MM6 cells observed between positive and negative controls increased with enhanced cell densities and starvation duration. The optimal cell density and starvation duration were determined to be 30'000 cells and 24 hour for the next experiments, respectively. The assay was successfully performed in 96-well plate format in 3 days instead of 4 days (Figure 7). In this experiment, it was suspected that there was a bias in cell counting which led us to investigate and develop a new staining procedure for accurate cell counting (see "New procedure for staining and counting HCAEC and MM6 cells using the Cellomics instrument" section).

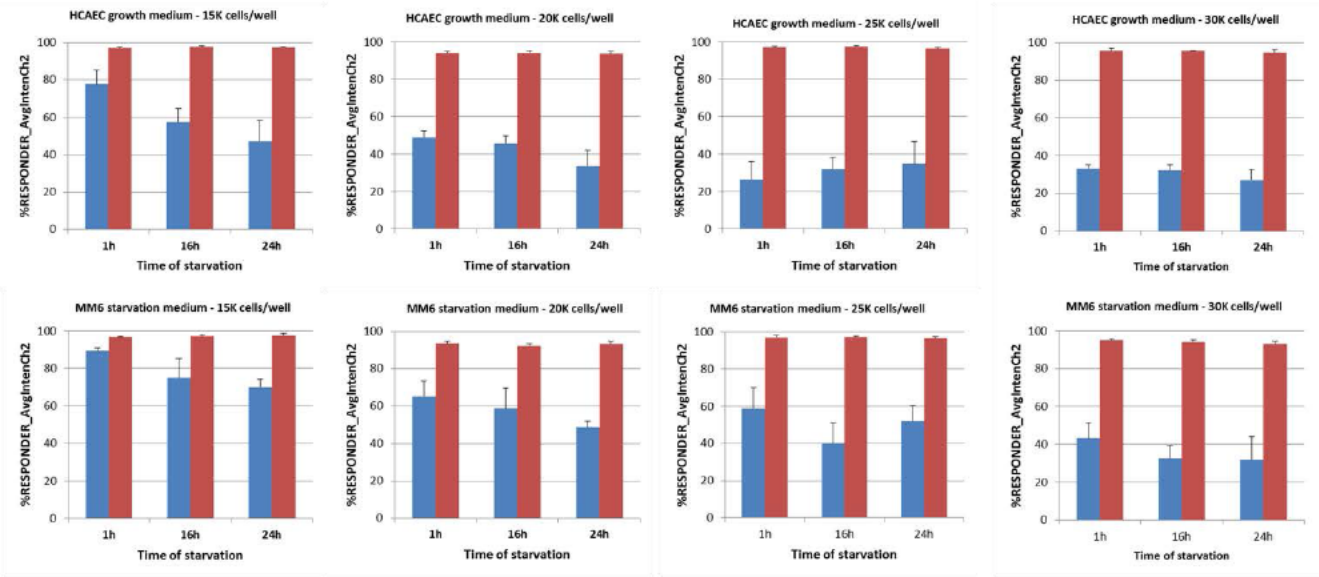


Figure 7: Effect of cell density, medium (HCAEC growth medium versus MM6 starvation medium) and time of starvation on the percentage of adherent MM6 cells relative to the total number of nuclei (previous staining and counting procedure) (Mean ± SD; N=1; n=4) (Exp035).

New procedure for staining and counting HCAEC and MM6 cells using the Cellomics instrument

In several experiments, the percentage of positive responders (corresponding to the adherent MM6 cells) was over-estimated by the Cellomics instrument in negative control conditions (see figure 7), if compared with an estimation done manually using the microscope. In addition, HCAEC cell count was observed to decrease while adherent MM6 cells were increasing (bias in cell counting). After investigated the protocol used by the Cellomics software that assumes a single cell-type population to count cells, which was not the case of the adhesion experiment using 2 different cell types, it was decided to modify the cell staining procedure to discriminate between co-cultured MM6 and HCAECs. A systematic experimental troubleshooting strategy testing different nuclear fluorescent dyes and fixative solutions enabled to obtain an accurate counting of both cell types separately. The key conditions to establish a reliable cell counting system using the Cellomics instrument were determined as follows:

- Nuclear staining of HCAECs with Hoechst and MM6 with Draq5 (blue and red fluorescent dyes, respectively).
- Fixing stained cells with 4% formaldehyde (after a period of 45 minutes corresponding to the incubation time of both cell types together to let the adhesion step occurring during the assay).
- Reading of the plate twice using specific filters of the Cellomics instrument: once for the Draq5 dye to count MM6 cells and once for the Hoechst dye to count HCAECs (Cellomics software protocols for: Draq5: "HCAEC-MM6 96w plate Reference levels Draq5" and for Hoechst: HCAEC-"MM6 96w plate Reference levels Hoechst").

Other fixative solutions such as glutaraldehyde (10%) and methanol (100%) were tested. It is important to note that the glutaraldehyde (10%) fixative solution interacted with Draq5 fluorescent dye leading to the detection of Draq5 fluorescence and the counting of Draq5-stained cells while using a specific filter to measure the fluorescence emitted by Hoechst dye (fluorescence bleeding). Fixation of MM6 cells followed by staining had an impact on the cell count which was lower than the count observed when cells were first stained and then fixed (Figure 8).

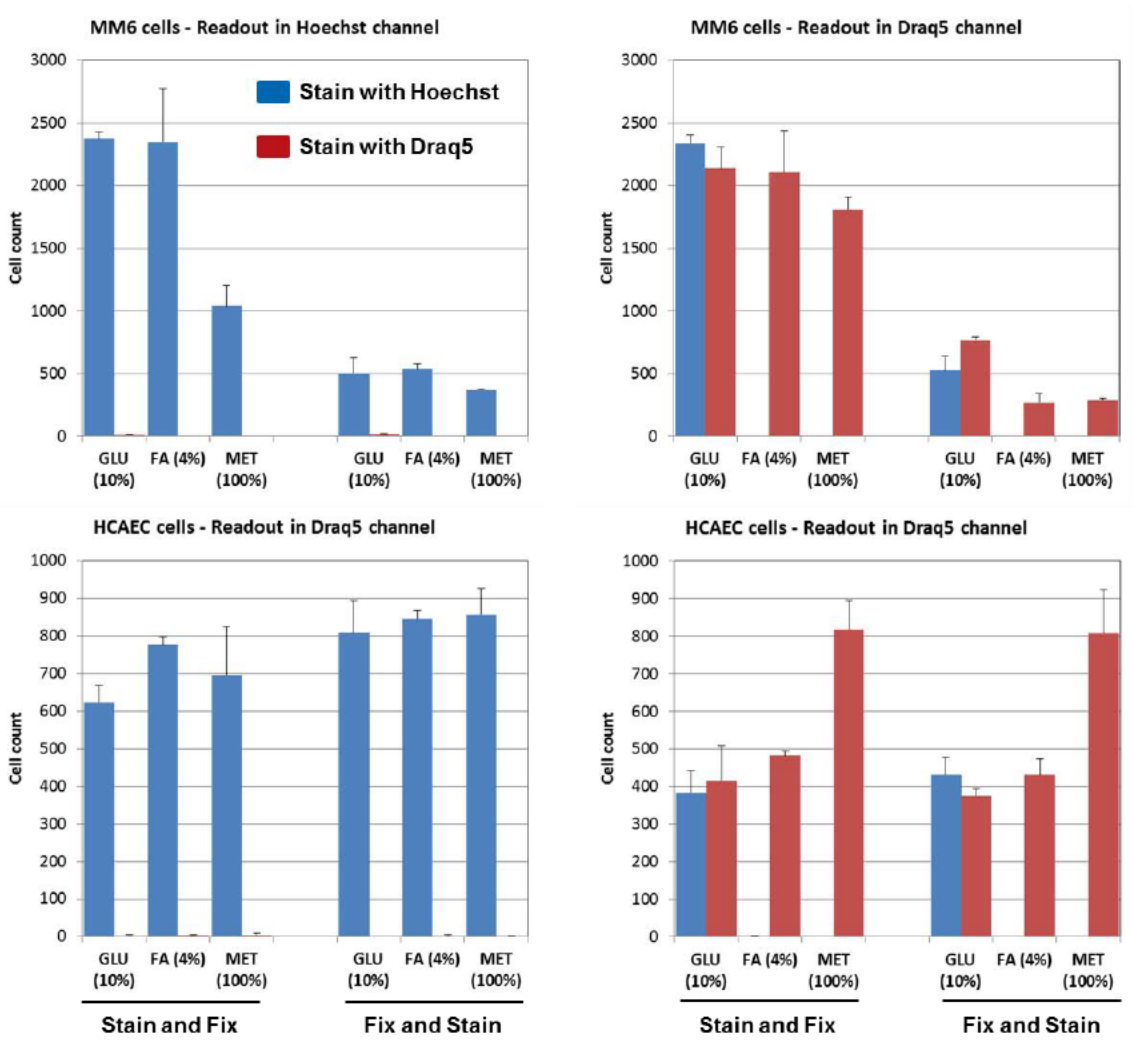


Figure 8: Effect of fixative solutions and staining/fixing sequences on count of MM6 and HCAEC cells stained with Hoechst and Draq5 nuclear dyes. Abbreviations: GLU: glutaraldehyde; FA: fomaldehyde; MET: methanol. (Mean ± SD; N=1; n=3) (Exp039)

Comments/Observations based on images

	Fixative solutions	Cell type	Channel	Hoechst staining	Draq5 staining
STAIN AND FIX	GLU (10%)	MM6	Hoescht		Cells can be seen, but few are counted (ghosts)
			Draq5	Cells not stained with Draq5 are counted in Draq5 channel	
		HCAEC	Hoescht		Cells can be seen, but are not counted (ghosts)
			Draq5	Cells not stained with Draq5 are counted in Draq5 channel	Many cells rejected
	FA (4%)	MM6	Hoescht		Cells can be seen, but are not counted (ghosts)
			Draq5	Cells are not detected at all (no ghost)	
		HCAEC	Hoescht		Cells are not detected at all (no ghost)
			Draq5	Cells are not detected at all (no ghost)	Many cells rejected
	MET (100%)	MM6	Hoescht	Many cells rejected. Effect of fixative?	Cells can be seen, but are not counted (ghosts)
			Draq5	Cells are not detected at all (no ghost)	Many cells rejected. Effect of fixative ?
		HCAEC	Hoescht		Cells are not detected at all (no ghost)
			Draq5	Cells are not detected at all (no ghost)	

Similar observations when the "FIX AND STAIN" procedure was followed. However, many less MM6 cells were counted compared to "STAIN AND FIX" procedure.

An experiment was performed to validate the new staining procedure. The results showed a significant correlation between the number of Draq5-stained MM6 cells added to the wells and the number of cells counted using the Cellomics instrument. The linear portion of the curve was observed between 10'000 and 100'000 Draq5-stained MM6 cells added to the wells. The number of Hoechst-stained HCAECs counted was similar across wells, and was not influenced by the number of Draq5-stained MM6 cells (Figure 9).

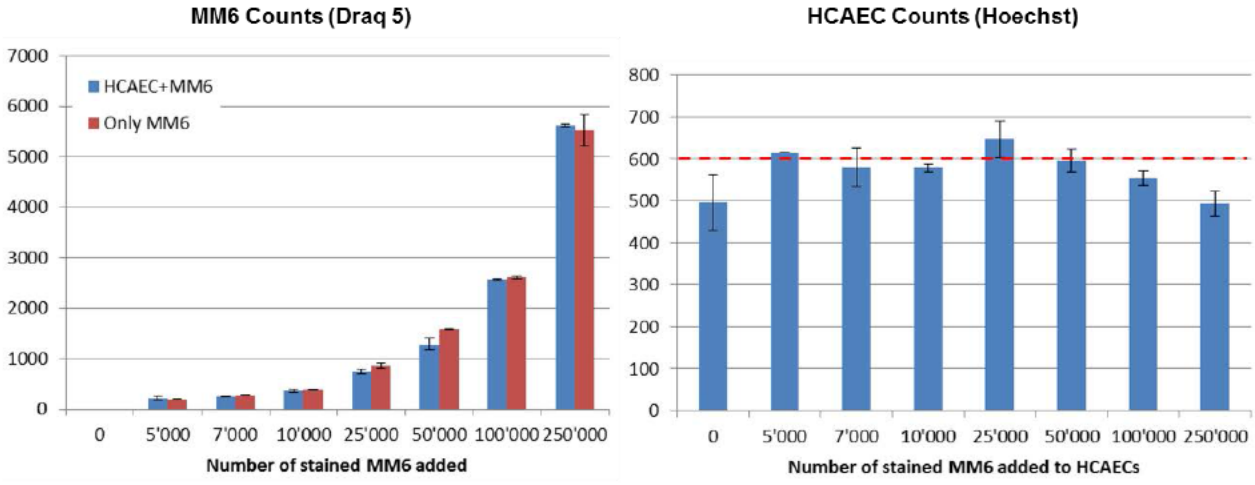


Figure 9: Draq5-stained MM6 and Hoechst-stained HCAEC counts when both cell types are incubated together or separately (Mean ± SD, N=1; n=4 for MM6 cells and n=2 for HCAECs; 6 fields/well) (Exp040).

Conditioned-medium preparation optimization with MM6 cells (for indirect treatment)

In the established protocol, conditioned medium preparation required the use of 4 million MM6 cells per well in a 6-well plate. To save precious MM6 cells and increase the number of possible tested conditions, some experiments have been conducted to optimize the preparation of conditioned-media with MM6 cells. Different number of MM6 cells (0.25, 0.5, 1 million MM6 per well) were seeded in 24-well plate and exposed to various concentrations of 3R4F sbPBS (HC) (0.03, 0.045, 0.06, 0.09, 0.12 puff/mL) diluted in MM6 starvation medium (0.5% FCS). LPS (1µg/mL) and MM6 starvation medium were used as positive and negative controls, respectively. TNFα concentrations were measured by ELISA in MM6 supernatant collected after 2 hour exposure (Figure 10).

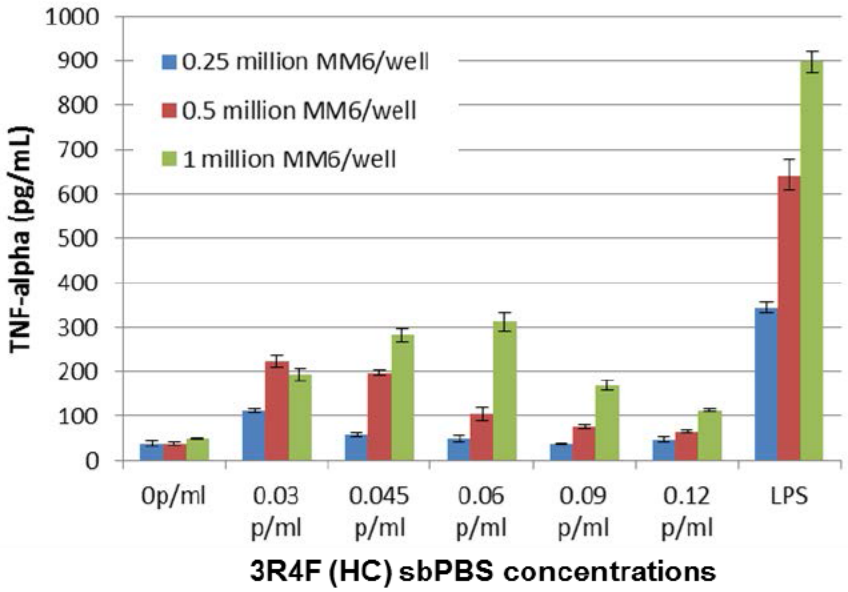


Figure 10: Effect of cell densities on TNFα produced by MM6 exposed to different concentrations of 3R4F sbPBS (HC) (Mean ± SD, N=1; n=3). (Exp037)

The results show a dose-dependent increase of TNFα in supernatant of MM6 exposed to 3R4F sbPBS (HC). TNFα levels positively correlated with increased number of MM6 cells. A maximum TNFα release (~300 pg/mL) was observed with 1 million MM6 cells per well at a dose of 0.06 puff/mL. The experiment was repeated twice increasing the number of MM6 cells to 2 million per well. The maximum of TNFα

concentration did not exceed the one observed with 1 million cells, however was reached at a dose of 0.09 puff/mL (Figure 11).

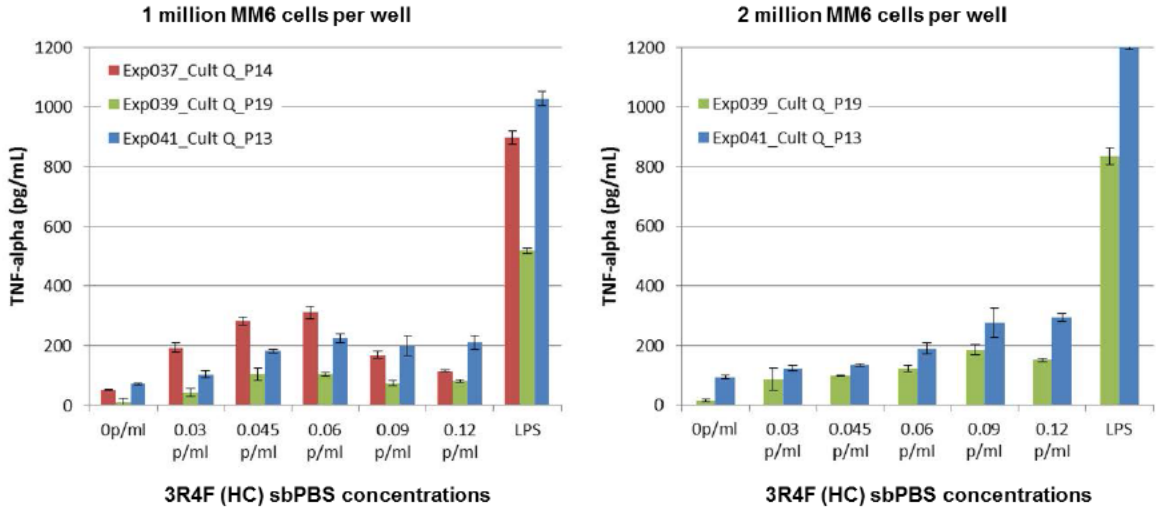


Figure 11: Results of 3 independent experiments on the effect of cell densities (1 and 2 millions) on TNFα produced by MM6 exposed to different concentrations of 3R4F sbPBS (HC) (Mean ± SD, Exp037: N=1; n=3; Exp039: N=1; n=2-3; Exp041: N=1; n=2-3).

In conclusion, the conditions chosen to generate conditioned media in future experiments will be one million MM6 cells per well (in 24-well plate) in a final volume of 1 mL.

Overall, the levels of TNFα present in supernatant of MM6 exposed to 3R4F sbPBS (HC) were 40 to 50% lower than the ones observed previously with 3R4F sbPBS (ISO) (in 6-well plate, 4 million MM6 cells in 4 mL).

Adhesion assay: sensitivity of HCAECs to TNFα

As we have previously shown that TNFα was an important factor present in sbPBS-treated MM6 supernatant that drives the adhesion of MM6 to HUVECs, it was important to assess the sensitivity of HCAECs to TNFα in the context of the adhesion assay. The treatment of HCAECs with enhanced doses of

TNFα (1, 10, 100, 250, 500, 1'000, 10'000 pg/mL) induced a dose-dependent increase of the number of MM6 cells bound to HCAECs (Figure 12).

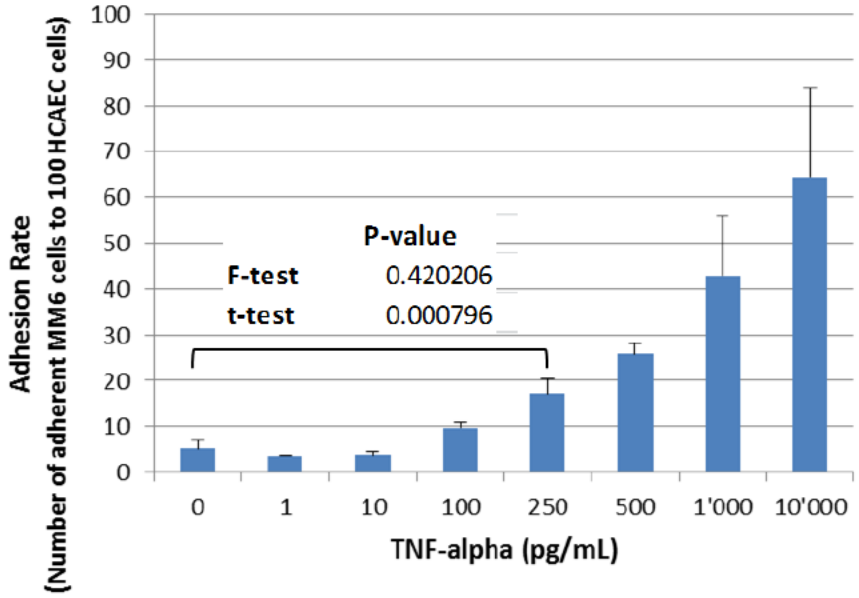


Figure 12: Dose-dependent effect of TNFα on the adhesion of MM6 cells to HCAECs (Mean ± SD; N=1; n=4; 16 fields/well) (Exp042)

A dose-dependent decrease of HCAEC cell index was observed in the first 5 hours following the exposure of HCAECs to increasing concentrations of TNFα. Then, HCAEC cell index re-increased close to initial values, suggesting a recovery to normal state of HCAECs (Figure 13).

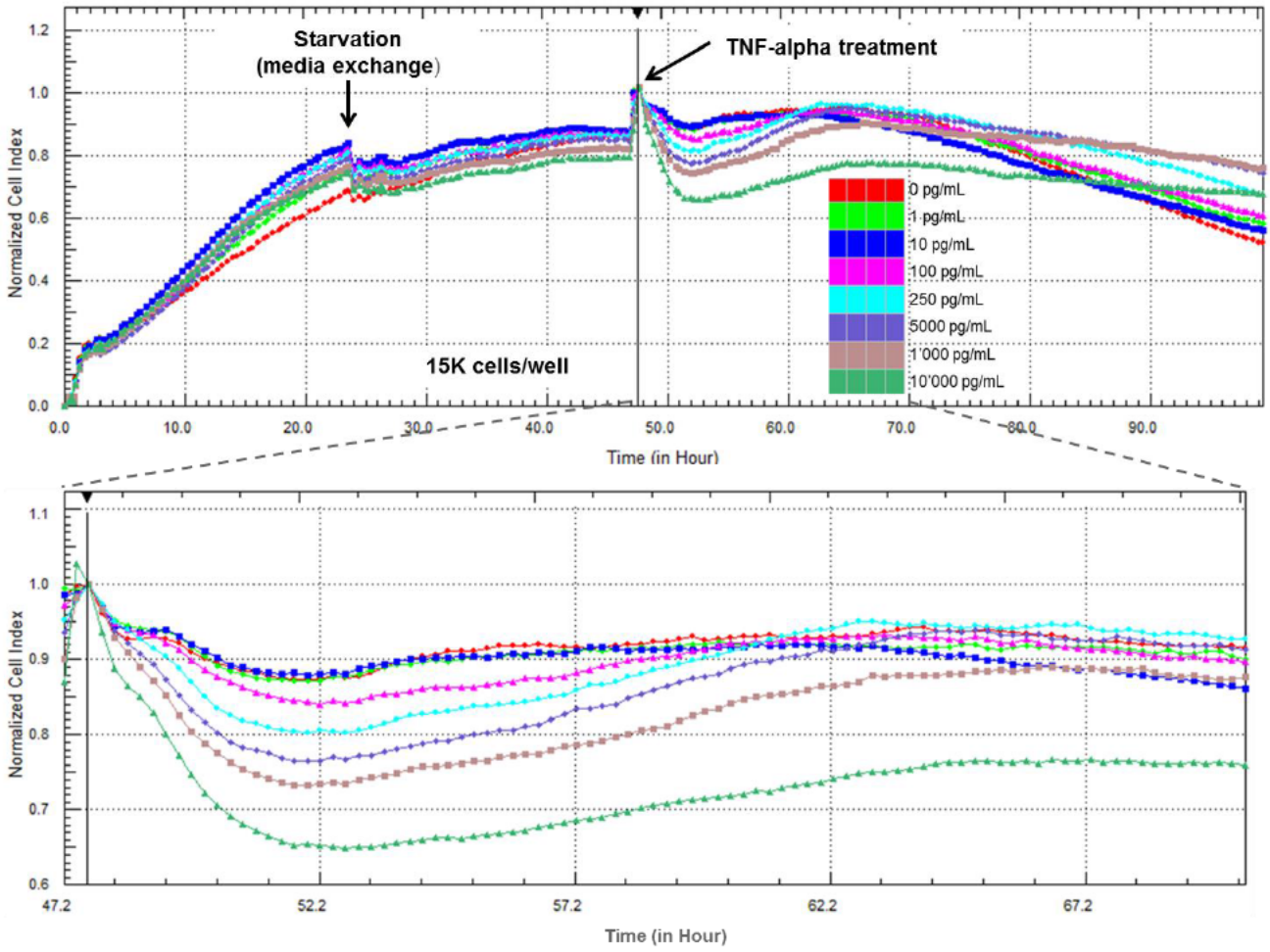


Figure 13: Effect of TNFα on HCAEC cell index over time. On day 1, 10 and 15K cells/well were seeded and grown for 24 hours in growth medium. On day 2, cells were starved (0.1% FCS) for 24 hours. On day 3, cells were exposed to different concentrations of TNFα (Exp042).

Adhesion assay in indirect and direct conditions using various concentrations of 3R4F sbPBS (HC)

The adhesion assay was conducted with enhanced concentrations of 3R4F sbPBS in direct and indirect conditions. The results are expressed as adhesion rate that corresponds to the number of adherent MM6 cells to 100 endothelial cells. In indirect conditions, the results show a dose-dependent increase of the adhesion of MM6 cells to HCAECs at a concentration of 0.045 puff/mL 3R4F sbPBS. Interestingly, the adhesion rate decreased at higher doses. In direct conditions, 3R4F sbPBS did not induce the adhesion of

MM6 cells to HCAECs. For all concentrations, similar adhesion rate (~6%) was similar to the one observed for the medium control (concentration: 0 puff/mL; basal adhesion) (Figure 14). Overall, the results suggest that soluble factors produced by MM6 cells exposed to 3R4F sbPBS (HC) promote the adhesion of MM6 cells to HCAECs. Although the adhesion rate profile remains the same across experiments, overall adhesion rate magnitudes can vary. One possible explanation could be the passage number at which MM6 cells are used for the assay. It seems that when cells are used at higher passages, overall adhesion rate magnitudes decrease.

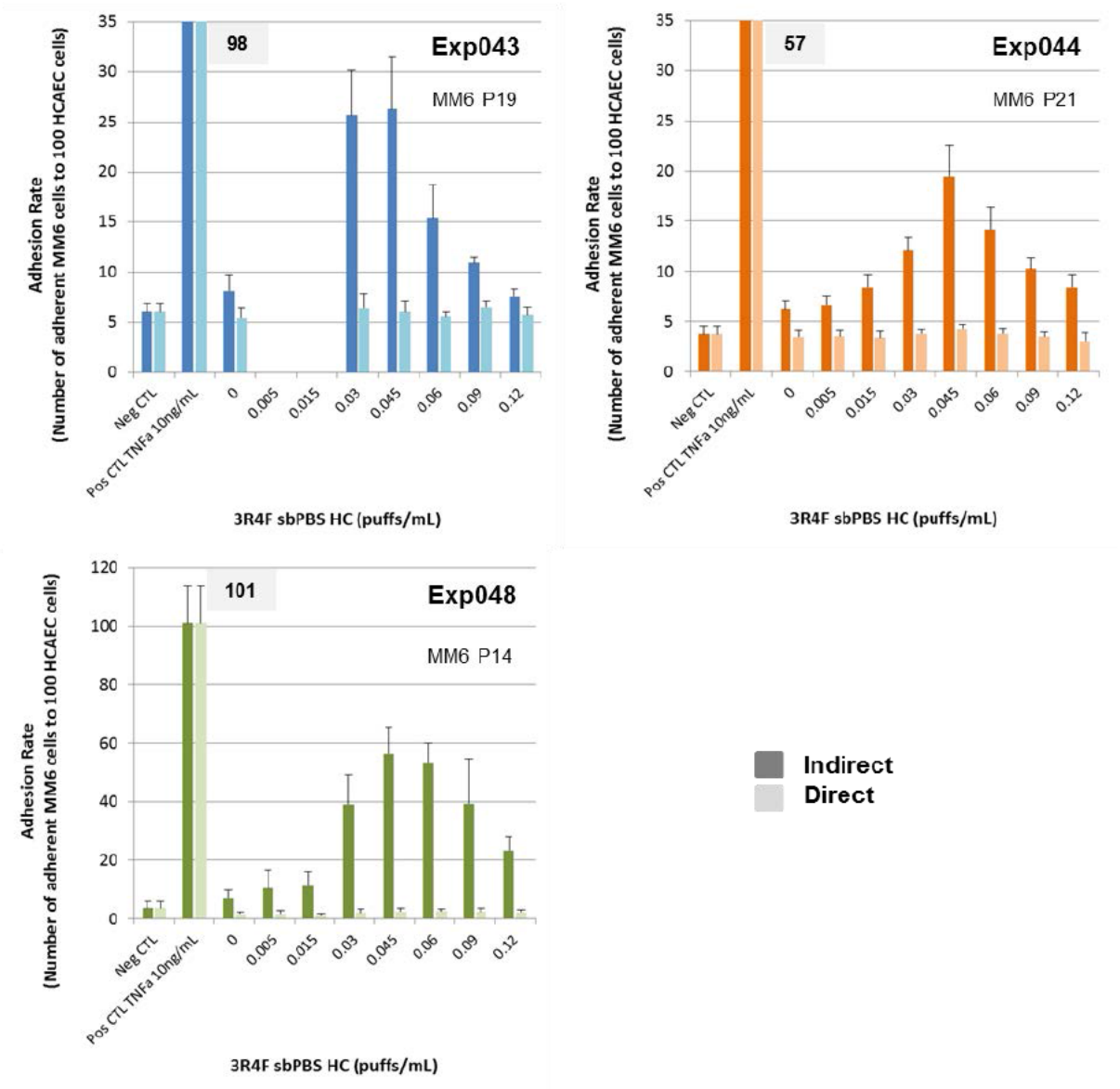
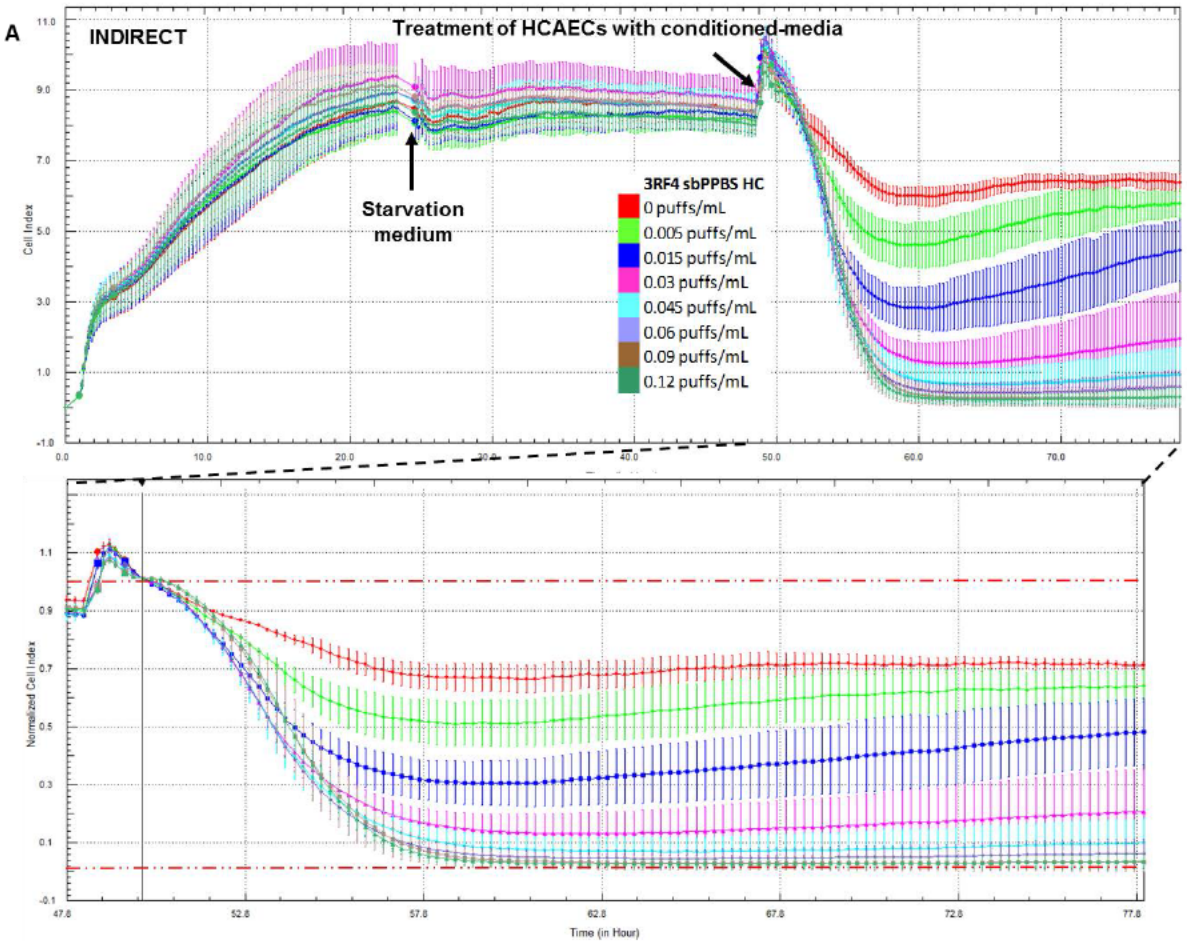


Figure 14: Dose-response of 3R4F sbPBS on the adhesion of MM6 cells to HCAECs. The adhesion rate was expressed as the number of MM6 cells bound to 100 HCAECs (Mean ± SD; N=3; n=4; 16 fields/well) (Exp043, Exp044 and Exp048).

The effect of conditioned-media on HCAECs was monitored measuring the cell index (impedance) over time. The results show a 3R4F sbPBS (HC) dose-dependent decrease of cell index values over time (Figure 15). The decrease reached a maximum after 10 hours treatment. The effect observed on HCAECs exposed to indirect and direct conditioned-media were similar suggesting a direct effect of smoke-derived components on HCAECs.



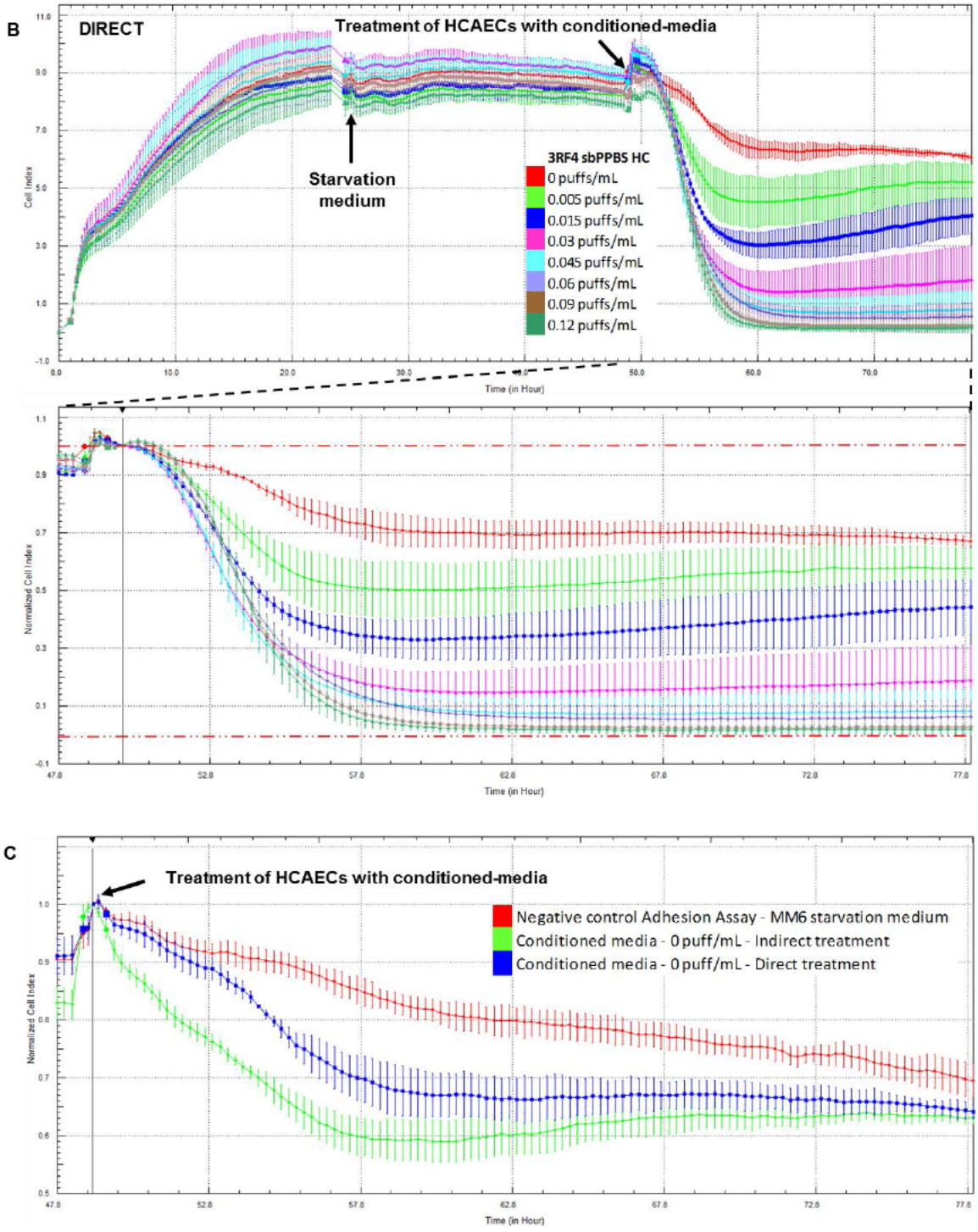

B
DIRECT
Treatment of HCAECs with conditioned-media
Starvation medium
3RF4 sbPPBS HC
0 puffs/mL
0.005 puffs/mL
0.015 puffs/mL
0.03 puffs/mL
0.045 puffs/mL
0.06 puffs/mL
0.09 puffs/mL
0.12 puffs/mL
Cell Index
Time (in Hour)
Normalized Cell Index
Time (in Hour)
C
Treatment of HCAECs with conditioned-media
Negative control Adhesion Assay - MM6 starvation medium
Conditioned media - 0 puff/mL - Indirect treatment
Conditioned media - 0 puff/mL - Direct treatment
Normalized Cell Index
Time (in Hour)

Figure 15: Effect of conditioned-media generated in indirect (A) and direct (B) conditions on HCAEC cell index over time. (C) Cell index curves related to negative controls (N=1; n=4; Exp045).

Cytokine level measurements in conditioned-media generated in indirect with 3R4F sbPBS (HC)

TNFα levels released in supernatant of MM6 cells exposed to different doses of 3R4F sbPBS (HC) increased in a dose-dependent manner reaching a plateau around 200 pg/mL at a dose of 0.045 puffs/mL, and then decreasing at highest doses (Figure 16). The TNFα levels measured were similar to the ones observed in Exp037, 039 and 041 (1 million MM6 cells per well) (Figure 11). The profile of TNFα levels correlated with the adhesion rate profile measured over doses of 3R4F sbPBS (Figure 14).

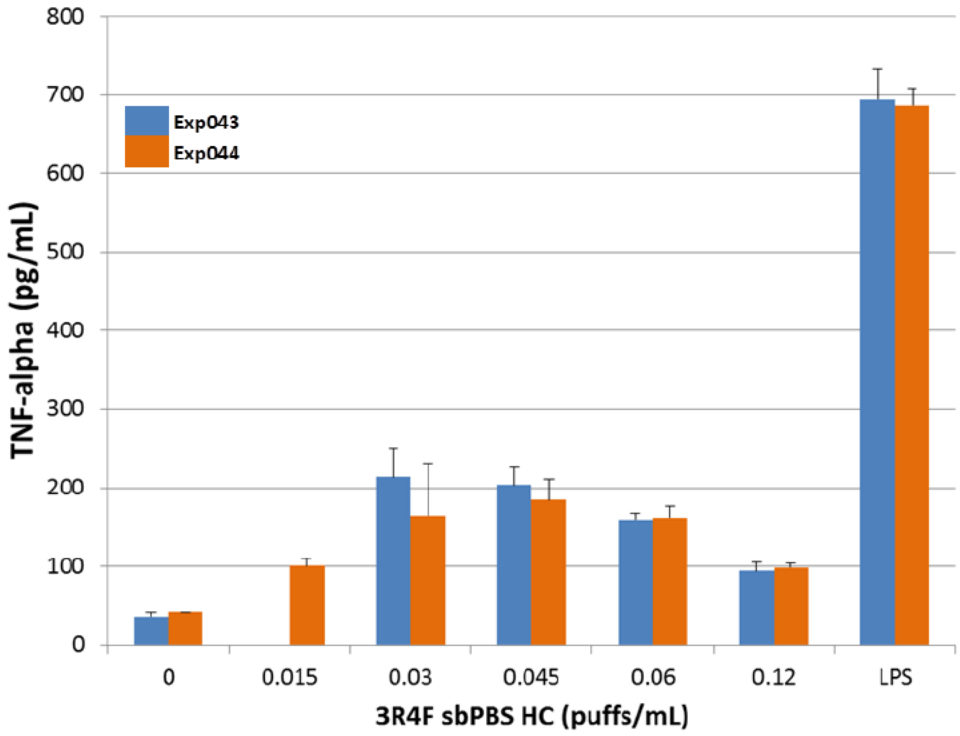


Figure 16: TNFα levels measured in conditioned-media generated in indirect conditions by ELISA (Mean ± SD; N=1; n=3; Exp046, samples from Exp043 and 44).

Other cytokines such as IL-12p70, IL-10, IL-6, IL-1β, IL-8 and also TNFα were measured in the same conditioned-media using the BD™ Cytometric Bead Array (CBA) kit (Exp046; samples from Exp043 and

044). However, the kit was expired since 6 months, and this experiment was considered as exploratory. The TNFα measurements followed the same profile as the one measured by ELISA (Figure 17). However, the measurements were of lower magnitude, suggesting a lower sensitivity and much more variable. IL-8 (chemotactic factor) release followed a similar profile to the one observed for TNFα, however much higher concentrations were measured (maximum of ~4500 pg/mL at 0.03 puffs/mL; Figure 17). No significant quantification was observed for the other cytokines. In future experiments, the results should be confirmed and/or improved using a new non-expired kit.

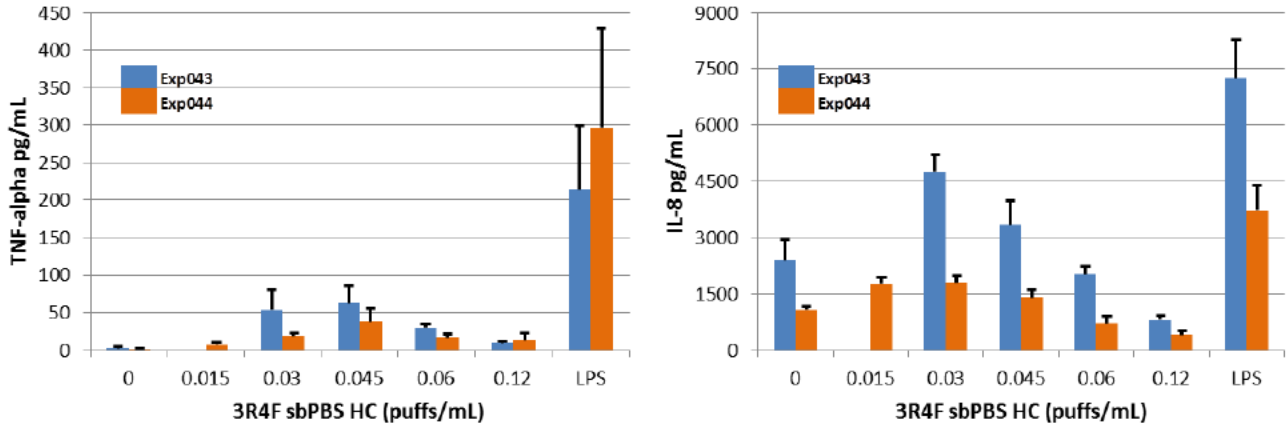
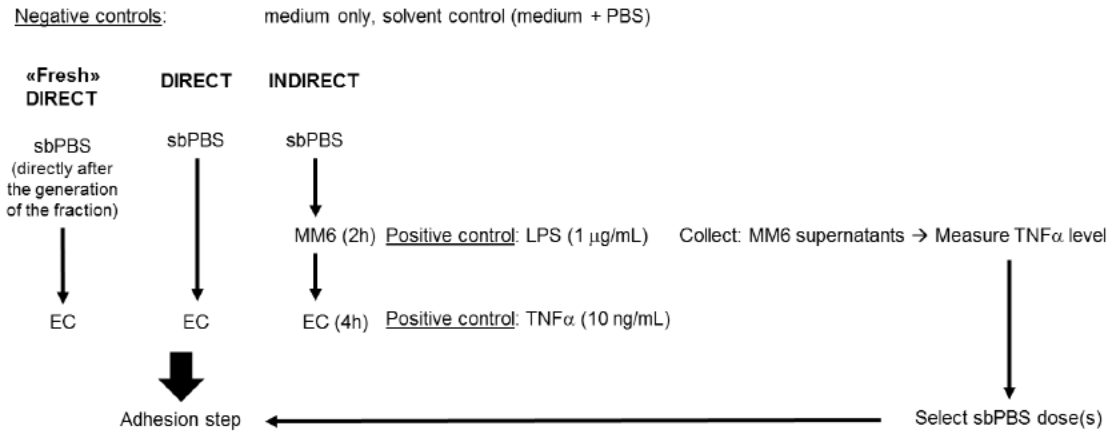


Figure 17: TNFα and IL-8 levels measured in conditioned-media generated in indirect conditions using the BD™ Cytometric Bead Array (CBA) kit (Mean ± SD; N=1; n=3; Exp046, samples from Exp043 and 44).

Adhesion assay in "Fresh" direct conditions using various concentrations of 3R4F sbPBS (HC)

The adhesion assay can be conducted in direct (Direct and "Fresh" Direct) or indirect treatment conditions.



In Direct protocol, the sbPBS medium is processed the same way as for the Indirect protocol in order to have a proper control, however without using MM6 cells (sbPBS is diluted in MM6 starvation medium, incubated for 2 hours at 37°C, frozen and thawed, incubated with HCAECs for 4 hours at 37°C). The sbPBS fraction contains volatile and highly reactive molecules among other CS constituents. The effect of sbPBS fraction on HCAECs might be different if the chemical content changes upon Direct and "Fresh" Direct protocols. Therefore, the adhesion assay has been also performed using the "Fresh" Direct protocol for which freshly generated sbPBS is rapidly used to treat HCAECs for 4 hours. Importantly, the results show an increased adhesion of MM6 cells to HCAECs at high sbPBS doses (0.09 and 0.12 puffs/mL) when using the "Fresh" Direct protocol (Figure 18). It will be important to confirm those results by repeating the experiment. For the same sbPBS doses, no significant adhesion was observed when using the Direct protocol (Figure 14).

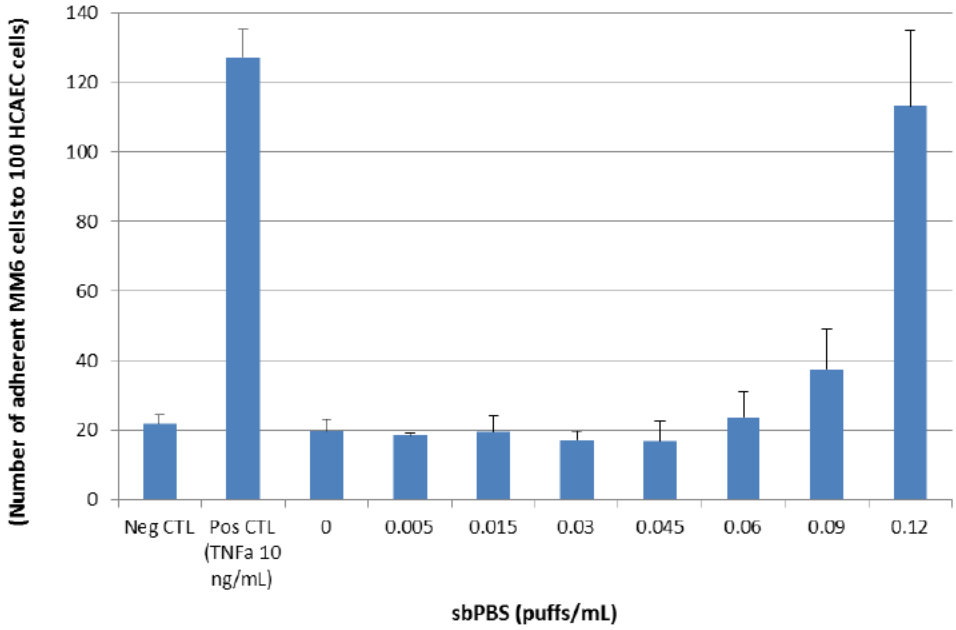


Figure 18: Dose-response of 3R4F sbPBS on the adhesion of MM6 cells to HCAECs performed in "fresh" direct conditions. The adhesion rate was expressed as the number of MM6 cells bound to 100 HCAECs (Mean ± SD; N=1; n=4; 16 fields/well) (Exp047).

In parallel to the adhesion assay, HCAECs cell index was recorded over time to evaluate the impact of "Fresh" sbPBS on HCAECs. A sbPBS dose-dependent decrease of the cell index was observed over time (Figure 19). Similar results were observed in Direct conditions, however with a different kinetic (Figure 15B) since sbPBS effect was much faster using the "Fresh" Direct compared to Direct protocols.

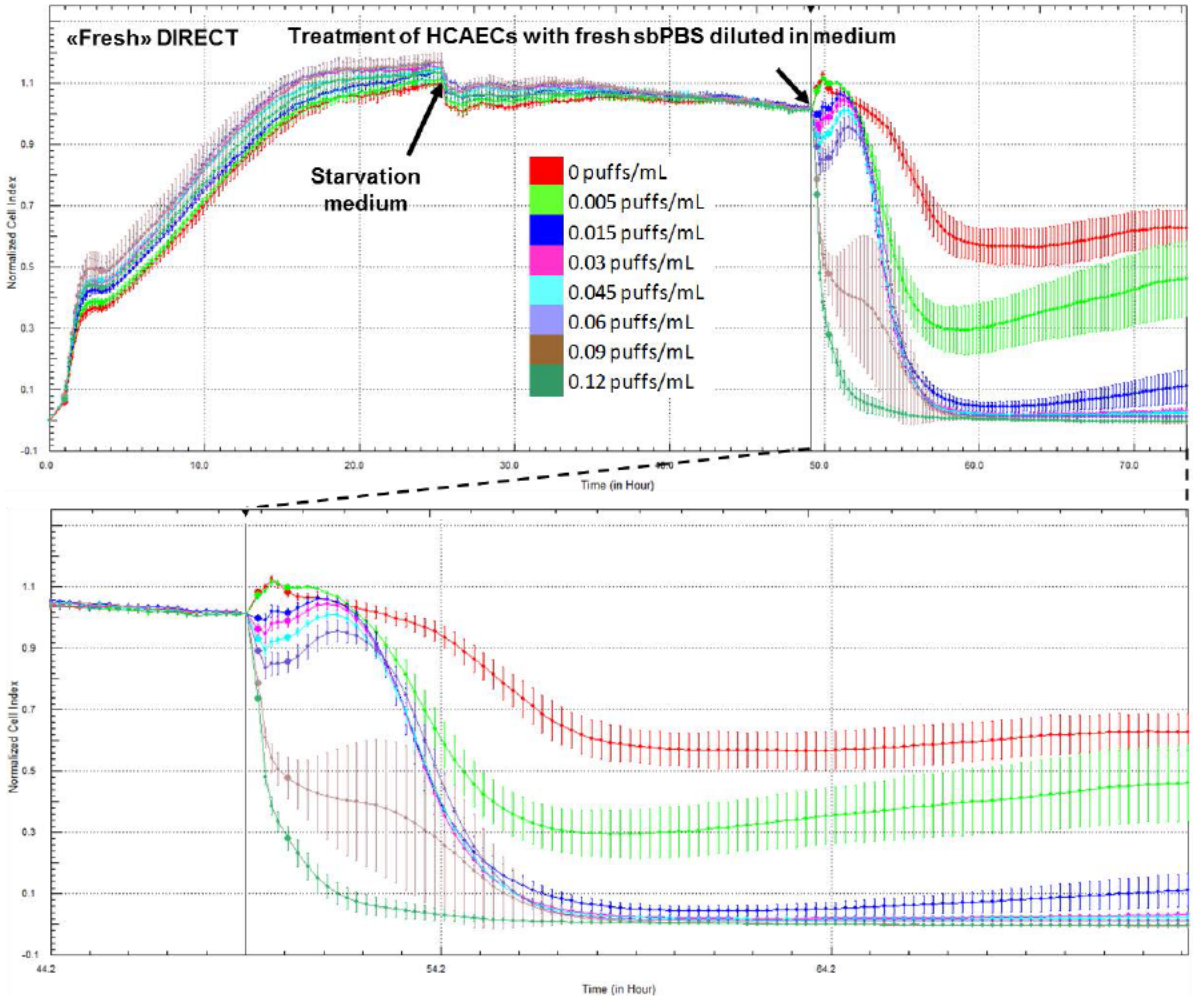


Figure 19: Dose-response of 3R4F sbPBS on HCAECs cell index over time. The experiment was performed using the "Fresh" Direct protocol (Exp047).

Chemical analysis of 3R4F sbPBS HC fractions

The table below contains the results of carbonyls analysis of 3R4F sbPBS fractions generated using the Health Canada smoking regimen protocol and used in the different experiments reported in the document:

Experiment	Week	Volume PBS (mL)	Items	Puff per item	FA	AA	AC	ACR	PA	CA	MEK	BA	Comment
Exp037	CW42	36	6	10.28	5.1	118.45	50.7	13.07	6.55	5.94	11.4	2.3	Figures 10 and 11
Exp039	CW44	36	6	9.90	4.47	154.55	53.3	15.61	7.48	5.68	13	2.41	Figures 10 and 11
Exp041	CW46	36	6	10.95	4.41	110.14	48.05	11.07	5.7	6.36	10.96	2.19	Figures 10 and 11
Exp043	CW49	36	6	10.57	3.59	130.27	47.39	9.78	4.46	6.11	12.37	1.37	Figures 14 and 16
Exp044	CW50	36	6	10.52	4.36	129.61	53.67	12.1	5.82	5.27	12.15	2.4	Figures 14 and 16
Exp045	CW51	36	6	10.68	4.07	120.5	55.47	13.32	5.19	5.5	13.9	1.86	Figure 15
Exp047	CW03	36	6	10.86	3.67	103.8	49.42	12.87	5.17	4.26	12.04	1.87	Figures 18 and 19
Exp048	CW04	36	6	12.06	3.11	84.48	38.98	9.72	3.94	3.73	8.94	2.06	Figure 14

Final concentration in microgram per puff

Formaldehyde	**FA**
Acetaldehyde	**AA**
Acetone	**AC**
Acrolein	**ACR**
Propionaldehyde	**PA**
Crotonaldehyde	**CA**
MEK	**MEK**
Butyraldehyde	**BA**

Summary table recapitulating optimal experimental parameters for the adhesion assay performed with HCAECs:

STEP	EXPERIMENTAL PARAMETERS
Preparation of conditioned-media	
Plate format	24-well plate
MM6 seeding density	10E6 cells per well in 0.5 mL
Starvation medium	MM6 culture medium with 0.5% FCS
Starvation period	2 hours
Exposure time (treatment)	2 hours
Final volume (treatment)	1 mL
Treatment of HCAECs with conditioned-medium	
Plate format	96-well plate
HCAEC seeding density	30'000 cells per well in 100 µL
Growth time after seeding	24 hours
Starvation medium	HCAEC culture medium MV2 with 0.1% FCS
Starvation period	24 hours
Exposure time (treatment)	4 hours
Final volume (treatment)	50 µL
Staining, adhesion and fixation	
MM6 nuclear staining	Draq5 (5 µM)
HCAEC nuclear staining	Hoechst (1 µg/mL)
Number of Draq5-stained MM6 cells added to HCAECs	250'000 cells
Time for adhesion	45 minutes
Time for fixation	15 minutes
Fixative solution	Formaldehyde 4%

Important experimental recommendations

- HCAECs used for an experiment should not exceed 4 passages.

- HCAECs are very sensitive to medium change, therefore full medium removal should be avoided for medium change steps. Only partial removal (70% of the volume) of the medium should be done.
- For the washing step after the incubation of MM6 cells with HCAECs (for the adhesion process), the plate has to be turned upside-down on a blotting paper to ensure the removal of all non-adherent MM6 cells. Otherwise, some non-adherent cells remained in the well and are counted as adherent MM6 cells.
- When the adhesion assay is performed in 48-well plate, a swirl effect can distribute non-homogeneously MM6 cells on the endothelial cell monolayer. This can bias the count of adherent cells depending on fields that are chosen for cell counting during image analysis.

Conclusions

An adhesion assay has been established and optimized with MM6 cells and HCAECs, more disease-relevant primary endothelial cells.

A new staining procedure has been established to count MM6 and HCAECs cells for the adhesion assay. This enables to calculate a more accurate and reliable adhesion rate.

A dose-dependent increase of the adhesion of MM6 cells to HCAECs reaching a maximum at 0.045 puffs/mL, then decreasing for higher doses was observed with the indirect protocol, while for the same range of doses, no significant adhesion was detected with the direct protocol. This observation suggests that the process of adhesion is driven by soluble factors released by MM6 cells in conditioned-media.

The release of TNFα by MM6 cells followed the same profile as the one observed for the adhesion rate over doses (increased up to 0.045 / 0.06 puffs/mL), and then, decreased for higher doses. Maximum TNFα concentrations ranged from 200 to 300 pg/mL across independent experiments.

Interestingly, a dose-dependent increase of the adhesion of MM6 cells to HCAECs was measured only at high doses when using the “Fresh” Direct protocol.

Irrespective of the protocol type (Indirect, Direct and "Fresh" Direct), a 3R4F sbPBS dose-dependent decrease of the HCAEC cell index was measured over time. However, the cell index drop was much faster in "Fresh" Direct treatment conditions compared to Direct and Indirect treatment conditions.

Overall, the results suggest that two different mechanisms drive the adhesion of MM6 cells to HCAECs. One mechanism goes through an indirect effect of 3R4F sbPBS at low/medium doses. Upon 3R4F sbPBS exposure, MM6 cells produce soluble factors (e.g. TNFα) that act on HCAECs to promote MM6 cell adhesion. The second mechanism goes through a direct effect of 3R4F sbPBS on HCAECs at high doses, however only when 3R4F sbPBS fraction is used to treat HCAECs just after it is generated. The results suggest that volatile and/or highly reactive compounds present in the sbPBS are responsible for the MM6 adhesion observed at high doses. Some remaining non-volatile/highly reactive CS constituents have an effect on HCAECs detected by a decrease of cell index over time, however do not promote the adhesion of MM6 cells to HCAECs.

Leveraging this optimized adhesion assay, future experiments are aimed at comparing the effects of MRTPs (THS2.2 and Yverdon) with 3R4F and identify appropriate doses to examine genome-wide gene expression data. We expect this approach to provide valuable insights into the mechanistic pathways affected by smoking of MRTPs and the magnitude of the effects in relation to 3R4F, the reference cigarette used across our studies.

Experimental Protocols

Culture of Human coronary artery endothelial cells (HCAECs)

Principle

The cultivation of HCAECs consists in growing cells and keeping them alive and healthy to be able to perform assays such as the adhesion assay.

Sample Requirements and Workload

HCAEC cells are maintained in the incubator at 37°C, 5%CO_2 and 70% relative humidity.

All the solutions (media, trypsin…) must be pre-warmed at 37°C in the water bath prior to use.

Material, Equipment, Chemicals, Standards and References

Cells:

Human Coronary Artery Endothelial Cells (HCAEC, lot 2121001.3) - Vitaris

Cell culture medium and reagents:

Growth medium: Endothelial Cell Growth Medium MV2 (Ready-to-use) – Vitaris - C-22022

Starvation medium: Endothelial Cell Growth Medium MV2 Kit – Vitaris - C-22121

Detachkit (Hepes/Trypsin/TNS) – Vitaris - C-41220-PRO

Penicillin/Streptomycin (100x) – Ruwag - P11-010

Specific consumables:

BD BioCoat™ Collagen I, 175cm2 – VWR - 734-0292

BD BioCoat™ Collagen I, 75cm2 – VWR - 734-0290

BD BioCoat™ Collagen I, 25cm2 – VWR - 734-0289

General consumables and reagents

Filter Stericup (0.22µm) – Fisher - W1582L

Phosphate Buffer Salt (PBS), without Ca^{2+}, Mg^{2+} – Sigma-Aldrich - D8537

Pipettes (5ml – 10ml – 25ml – 50ml)

Falcon tubes (15ml – 50 ml)

Pipettes and pipette tips (10µl – 200µl – 1000µl)

Centrifuge

Incubator

Cell counter (e.g. Casy)

Biological hood

Gloves

Procedure

Sample Collection

Samples (frozen cells) are collected from the liquid nitrogen dewar.

Sample Preparation

N/A

Setting Up of Instruments

Casy Cell Counter - HCAEC protocol settings:

Aggr Correction: 1500. fl

Evaluation Cursors: 11.38 to 50µm

Normalization Cursors: 7.47 to 50µm

Preparation of Solutions and Media

PromoCell Endothelial Cell Growth Media MV / MV2 are available as Medium (Ready-to-use) or as Medium Kit. The Medium (Ready-to-use) consists of a 500 ml bottle of Basal Medium and one vial of SupplementMix. The Medium Kit consists of a 500 ml bottle of Basal Medium and the SupplementPack (a set of individual vials with pre-measured supplements) allowing the user full control over the media formulation. Adding the SupplementMix or the SupplementPack to the Basal Medium results in the complete Growth Medium. Basal Medium as well as SupplementMix and SupplementPacks can also be purchased separately.

HCAEC growth medium (2% FBS):

Volume	%	Medium/Reagent	Supplier/Ref number
500ml		Endothelial Cell Growth Medium MV2 (Ready-to-use) (consists of a 500 ml bottle of Basal Medium and one vial of SupplementMix)	Vitaris - C-22022
5ml	1%	Pen/Strep	PAA - P11-010

→Filter the medium 0.22µm

HCAEC starvation medium (0.1% FBS):

Volume	%	Medium/Reagent	Supplier/Ref number
500ml		Endothelial Cell Growth Medium MV2 Kit The Medium Kit consists of a 500 ml bottle of Basal Medium and the SupplementPack (a set of individual vials with pre-measured supplements). Add all the content of the supplement vials, excepted the serum (FCS), **add only 1.25ml** to have 0.1% serum medium	Vitaris - C-22121
5ml	1%	Pen/Strep	PAA - P11-010

→Filter the medium 0.22µm

Number of determinations

N/A

Daily verification or according to use

Every time before using cells, the culture is checked under an inverted microscope to see if cells are growing correctly and are free of bacteria/fungi contaminations.

Testing Procedure

Thawing:

Take a vial out from the liquid nitrogen and decontaminate it with 70% ethanol.

Under the laminar flow, quickly open and close the vial cap.

Place the vial in the water bath at 37°C and check the thawing of the cells.

As soon as the vial is thawed, decontaminated it and transfer the cell suspension (1ml) in 9ml of pre-warmed growth medium.

Centrifuge 5' at 300g at room temperature.

Re-suspend the cell pellet in 10ml growth medium.

Count the cells: 100µl cells in 9.9ml CasyTon.

Seed all the content in a T175 Collagen A coated flask and add 25ml growth medium.

Split the cells 3 or 4 days later.

Split:

Pre-warm the DetachKit (Hepes + Trypsin/EDTA + TNS) and the growth medium.

Wash the cells 2 times with 6ml of Hepes solution (volume for a T175 flask).

Add 6ml of Trypsin/EDTA and let the flask at room temperature for 2min.

Visually check the detachment of the cells with the inverted microscope.

Stop the trypsin with 6ml TNS and collect the cells in a Falcon tube.

Centrifuge 5min at 300g.

The supernatant is carefully removed and the cell pellet is re-suspended in 10ml growth medium.

Count the cells: 100µl cells in 9.9ml CasyTon.

Seed the cells according to your needs.

Flask format	Volume	Seeding for 3 days	Seeding for 4 days
T75	≈ 15 - 20ml	800'000 cells/flask	400'000 cells/flask
T175	≈ 35 – 40ml	1'200'000 cells/flask	1'000'000 cells/flask

Freezing:

Prepare freezing medium: 70% growth medium + 20% FBS + 10% DMSO

Count and centrifuge the cells.

Re-suspend the cell pellet in freezing medium to have a cell concentration of 1E6 cells/ml and seed 1ml/vial.

Put the vials into a freezing boxe and place it at -80°C for 24h. Then, the vials could be stored in the liquid nitrogen dewar.

Culture of mono mac 6 cells (MM6)

Principle

The cultivation of MM6 consists in growing cells and keeping them alive and healthy to be able to perform assays such as the adhesion assay.

Sample Requirements and Workload

MM6 cells are maintained in the incubator at 37°C, 5%CO2 and 70% relative humidity.

All the solutions (media, trypsin…) must be pre-warmed at 37°C in the water bath prior to use.

Material, Equipment, Chemicals, Standards and References

Cells:

MM6 cells – DSMZ - ACC 124

Cell culture medium:

RPMI 1640 – PAA - E15-039

OPI Media Supplement - Sigma - O5003

FBS low LPS – PAA - A15-102

L-Glutamine (200mM) – PAA - M11-004

NEAA (100x) – PAA - M11-003

Penicillin/Streptomycin (100x) – Ruwag - P11-010

Specific consumables:

Cell culture plates for suspension cells 24-well – Sarstedt - 83.1836.500

General consumables and reagents

Filter Stericup (0.22µm) – Fisher - W1582L

Phosphate Buffer Salt (PBS), without Ca^{2+}, Mg^{2+} – Sigma-Aldrich - D8537

Pipettes

Falcon tubes

Centrifuge

Incubator

Cell counter (e.g. Casy)

Biological hood

Gloves

Procedure

Sample Collection

Samples (frozen cells) are collected from the liquid nitrogen dewar.

Sample Preparation

N/A

Setting Up of Instruments

Casy Cell Counter - MM6 protocol settings:

Aggr Correction: Auto

Evaluation Cursors: 9.61 to 60µm

Normalization Cursors: 5.98 to 60µm

Preparation of Solutions and Media

MM6 growth medium (10% FBS):

Volume	%	Medium/Reagent	Supplier/Ref number
500ml		RPMI 1640	PAA - E15-039
10ml	1.7%	Pen/Strep	PAA - P11-010
5ml	0.86%	OPI media supplement (reconstituted in 10ml MilliQ water)	Sigma - O5003
5ml	0.86%	L-Glutamine	PAA - M11-004
5ml	0.86%	NEAA	PAA - M11-003
58ml	10%	FBS low LPS	PAA - A15-102

→Filter the medium 0.22µm

MM6 starvation medium (0.5% FBS):

Volume	%	Medium/Reagent	Supplier/Ref number
500ml		RPMI 1640	PAA - E15-039
10ml	1.7%	Pen/Strep	PAA - P11-010
5ml	0.86%	OPI media supplement (reconstituted in 10ml MilliQ water)	Sigma - O5003
5ml	0.86%	L-Glutamine	PAA - M11-004
5ml	0.86%	NEAA	PAA - M11-003
2.5ml	0.5%	FBS low LPS	PAA - A15-102

→Filter the medium 0.22µm

Number of determinations

N/A

Daily verification or according to use

Every time before using cells, the culture is checked under an inverted microscope to see if cells are growing correctly and are free of bacteria/fungi contaminations.

Testing Procedure

Thawing:

Take a vial out from the liquid nitrogen and decontaminate it with 70% ethanol.

Under the laminar flow, quickly open and close the vial cap.

Place the vial in the water bath at 37°C and check the thawing of the cells.

As soon as the vial is thawed, decontaminated it and transfer the cell suspension (1ml) in 9ml of pre-warmed growth medium.

Centrifuge 7' at 400g at room temperature.

Re-suspend the cell pellet in 10ml growth medium.

Count the cells: 100µl cells in 9.9ml CasyTon.

Adjust cell suspension to 2E5 cells/ml and seed 2ml/well in a 24w plate (= 4E5 cells/well).

Split the cells 3 or 4 days later.

Split:

Mix carefully the MM6 cells and pool the wells with a 5ml pipette.

Count the cells: 100µl cells in 9.9ml CasyTon.

Adjust the cell suspension and seed 2ml/well in a 24w plate.

Friday → Monday - 3 days: Cell suspension 2E5 cells/ml (4E5 cells/well)

Monday → Friday - 4 days: Cell suspension 1E5 cells/ml (2E5 cells/well)

Add 500µL/well fresh growth medium on Wednesday

When a wash is required, to re-suspend the cells in starving medium for example, centrifuge the cells 7' at 400g.

Re-suspend slowly the pellet with a 1000µL Eppendorf pipette in 1ml of medium.

Then, add the required volume and gently mix the cells.

Freezing:

Prepare freezing medium: 70% growth medium + 20% FBS + 10% DMSO

Count and centrifuge the cells.

Re-suspend the cell pellet in freezing medium to have a cell concentration of 5E6 cells/ml and seed 1ml/vial.

Put the vials into a freezing boxe and place it at -80°C for 24h. Then, the vials could be stored in the liquid nitrogen dewar.

Adhesion assay

Day 1:

HCAEC cells seeding:

Prepare a cell suspension at 300'000 cells/mL in growth medium and seed 100 µL/well in a 96w plate coated with collagen A (= 30'000 cells/well). Incubate the plate at 37°C for 24h.

MM6 cells seeding:

Prepare a cell suspension at 200'000 cells/mL in growth medium and seed 2 mL/well in a 24w plate for cell suspension (= 400'000 cells/well). Adjust the volume to prepare according to your needs. Cells will be ready for Day3.

Generation of conditioned media **with** MM6 cells – Indirect treatment:

Prepare a cell suspension at 2 million MM6 cells/mL in starvation medium and seed 0.5 mL/well in a 24w plate for suspension cells (=1million MM6/well). Incubate 2h at 37°C. Fifteen minutes before the end of the incubation time, prepare the sbPBS dilution in MM6 starvation medium and following the dosing sheet.

At the end of the incubation time, add 0.5 mL/well of diluted sbPBS to be tested (0 to 0.15 puff/mL – 3 to 4 replicates – See dosing sheet) or triton (10% final) for the LDH positive control and LPS (1µg/mL final) for ELISA positive control. Incubate 2h at 37°C and collect the cells in 15 mL Falcon tube. Centrifuge 7min at 400g and collect the supernatants (=conditioned media) in 2 separate tubes or plates (300 µL for adhesion assay + 300 µL for ELISA and LDH). Store the samples at -80°C until the adhesion assay experiment on Day3.

Generation of conditioned media **without** MM6 cells – Direct treatment:

Put 0.5 mL of starvation medium in a 24w plate for suspension cells and incubate 2h at 37°C.

At the end of the incubation time, add 0.5 mL/well of diluted sbPBS to be tested (0 to 0.15 puff/mL – 3 to 4 replicates – See dosing sheet) or LPS (1µg/mL final) for ELISA positive control. Incubate 2h at 37°C and collect the cells in 15 mL Falcon tube. Centrifuge 7min at 400g and collect the supernatants (=conditioned media) in tubes or plates. Store the samples at -80°C until the adhesion assay experiment on Day3.

Preparation of the ELISA plate (if ELISA analysis required):

Thaw the capture Ab (4µg/mL) and add 100 µL/well in a 96w plate (white with clear bottom). Seal the plate and incubate overnight at room temperature.

Day 2:

Endothelial cells starvation:

Remove 75 µL/well of growth medium and add 100µL/well of HCAEC starvation medium (0.1%FCS). Incubate for 24h at 37°C.

ELISA (If required)

Aspirate each well and wash 3 times with 300 µL washing buffer. Block the plate by adding 300 µL/well of reagent diluent and incubate at least 1 hour at room temperature.

During the incubation time, prepare TNFα standard dilutions (2-fold serial dilutions - High standard at 1000 pg/mL).

Tube n°	[TNFα]	Dilution
8	1000	27 µL TNFα stock (370ng/mL) + 10 mLReagent diluent
7	500	0.5 mL Reagent Diluent + 0.5 mL previous dilution
6	250	0.5 mL Reagent Diluent + 0.5 mL previous dilution
5	125	0.5 mL Reagent Diluent + 0.5 mL previous dilution
4	62.5	0.5 mL Reagent Diluent + 0.5 mL previous dilution
3	31.25	0.5 mL Reagent Diluent + 0.5 mL previous dilution
2	15.63	0.5 mL Reagent Diluent + 0.5 mL previous dilution
1	0	0.5 mL Reagent Diluent

At the end of the incubation time, aspirate each well and wash 3 times with 300 µL of washing buffer. Add 100 µL/well of sample or standard. Cover with an adhesive strip and incubate 2 hours at room temperature. Wash 3 times (300 µL/well) and add 100 µL/well of detection Ab. Cover with a new adhesive strip and incubate 2 hours at room temperature.

Prepare Streptavidin-HRP with dilution 1:200 in reagent diluent.

Wash 3 times (300 µL/well) and add 100 µL/well of Streptavidin-HRP working solution. Cover the plate and incubate for 20min at room temperature protected from the light. Prepare the Substrate solution Color reagent A + Color reagent B (1:1). Wash 3 times (300 µL/well) and add 100 µL/well of the Substrate solution (becomes blue). Cover the plate and incubate for 20min at room temperature. Protect from light. Add 50 µL/well of Stop solution (become yellows) and gently tap the plate to ensure thorough mixing. Determine the optical density at 450nm and 540nm using the Fluostar Optima (Program TNFα).

LDH assay (If required):

Equilibrate Substrate Mix and Assay Buffer to room temperature.

Prepare CytoTox-ONE Reagent by adding 11 mL of Assay Buffer to the Substrate Mix and protect the reagent from direct light.

Controls:

Untreated cells control: Untreated supernatant as vehicle control

Maximum LDH release control:

Treat cells with Triton 10% final (diluted in MM6 starving medium) for 2 hours

Collect and centrifuge the cells

Collect supernatant

Add 100 µL/well of sample (MM6 supernatants), equilibrate plate to RT and add 100 µL/well of CytoTox-One Reagent. Shake the plate for 30 sec and incubate at RT for 10 min. Add 50 µL/well of Stop Solution, shake the plate for 10 sec and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm.

Day 3:

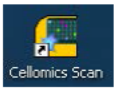
Perform the adhesion assay:

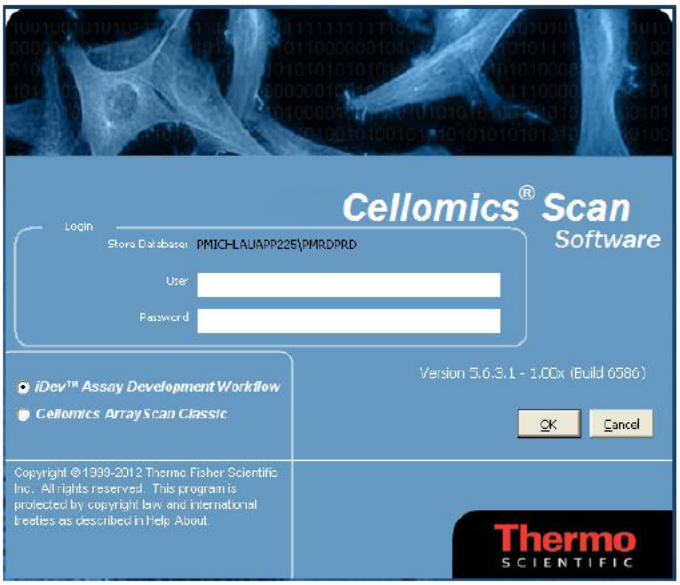
Timing/Steps	Procedure
8:30 – 9:30 **Samples preparation**	Thaw conditioned media – Prepare positive control (TNFα, 10ng/mL diluted in MM6 starvation medium) and negative control (MM6 starvation medium with 7.5%PBS)
9:30 - 9:45 **Samples addition**	Remove carefully 90 µL/well of media from the 96w plate seeded with HCAECs on Day1– Add 50 µL/well of sample to be tested (conditioned media and controls) onto the HCAECs cells – Incubate at 37°C for 4h
12:45 – 14:00 **MM6 staining**	Pool, count and centrifuge MM6 cells prepared on Day1 Re-suspend the pellet in MM6 starvation medium with 5µM Draq5 to have a cell suspension at ≈10E6 cells/mL - Incubate 15min at 37°C Centrifuge 7min at 400g and wash twice with PBS Re-suspend at 5E6 cells/mL in MM6 starvation medium
13:45 **HCAEC staining**	At the end of the 4h incubation, remove carefully 40 µL/well, add 100 µL/well of PBS and remove 100 µL Add 50 µL/well of Hoechst (1µg/mL) – Incubate 15min at 37°C Wash twice with PBS
14:00 – 14:45 **Addition of MM6 to HCAECs**	Add 50 µL/well of stained MM6 onto the HCAECs – Incubate 45min at 37°C
14:45 -15:00 **Fixation**	Wash 3 times with PBS Add 50 µL/well of Formaldehyde 4% diluted in PBS - Incubate 15min at 37°C Wash once with PBS and re-fill with 100 µL/well PBS

15:00 – 17:30 **Plate reading**	Read the plate with the Cellomics ArrayScan using Draq5 channel to count MM6 cells and Hoeschst channel for the HCAECs. See the procedure below. Note: The plate can be stored 24h at 4°C protected from light for a later readout

Procedure to read the plate(s) with the Cellomics ArrayScan:

- Open the software "Cellomics Scan"



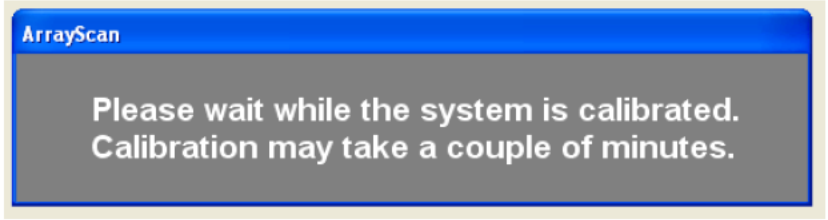


- Login yourself:

User: your own PMI login

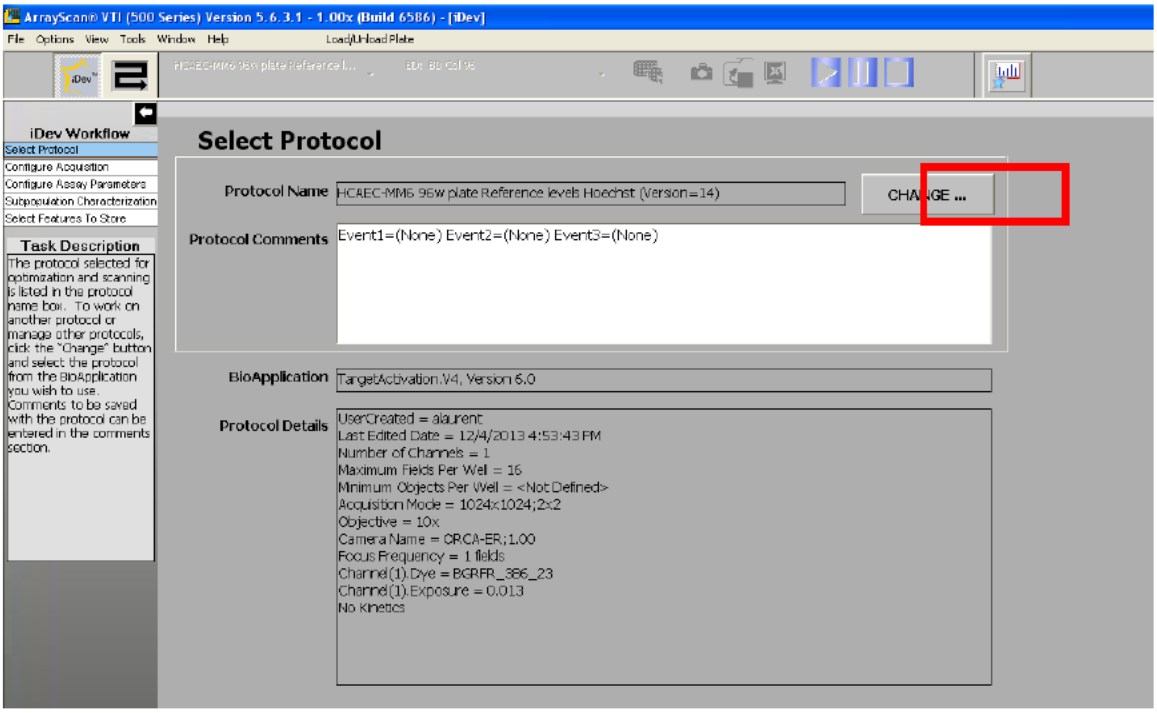
Password: (b) (4)

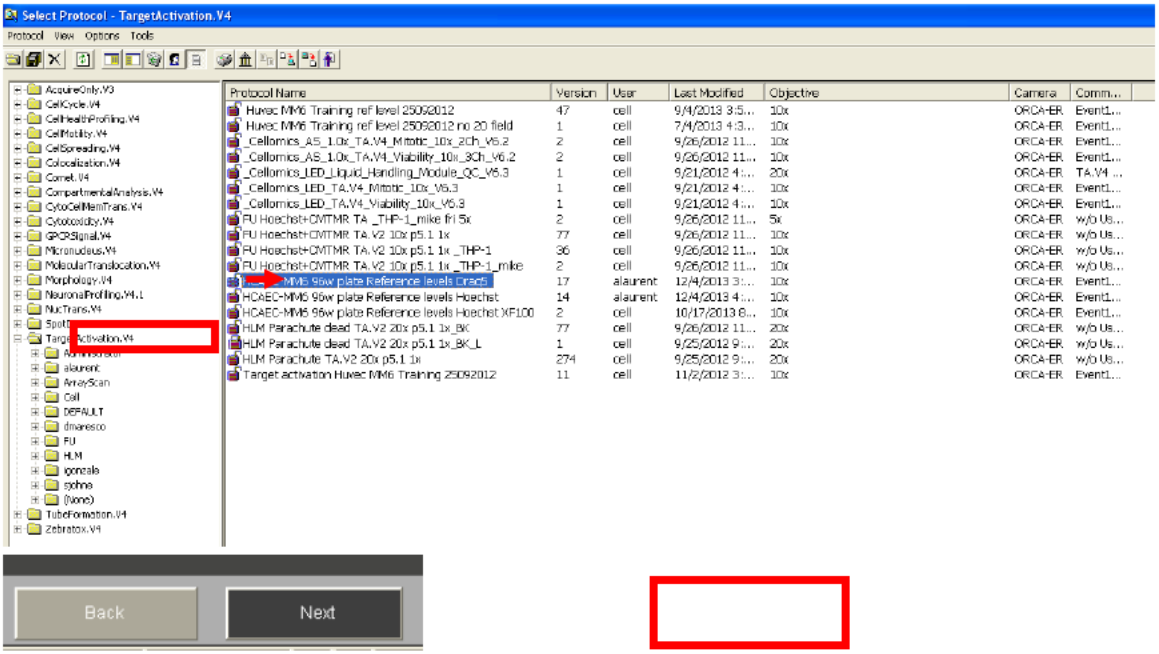
- Load the plate in the instrument



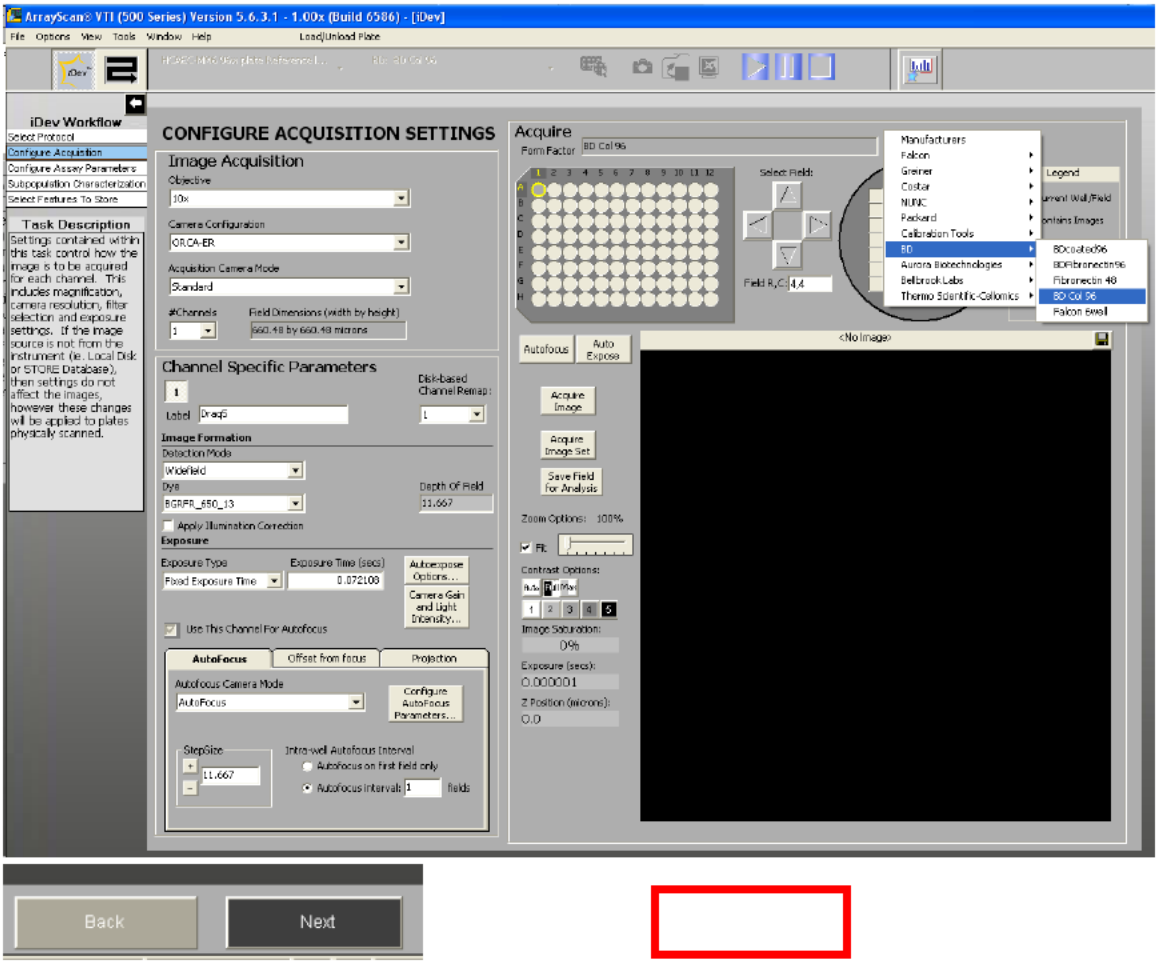
- Select the appropriate protocol:

- Click on “Change”
- Choose “Target Activation V4”
- Select the protocol “HCAEC-MM6 96w plate Reference levels Draq5” to count the MM6 cells
- Click on “Next” at the bottom of the page

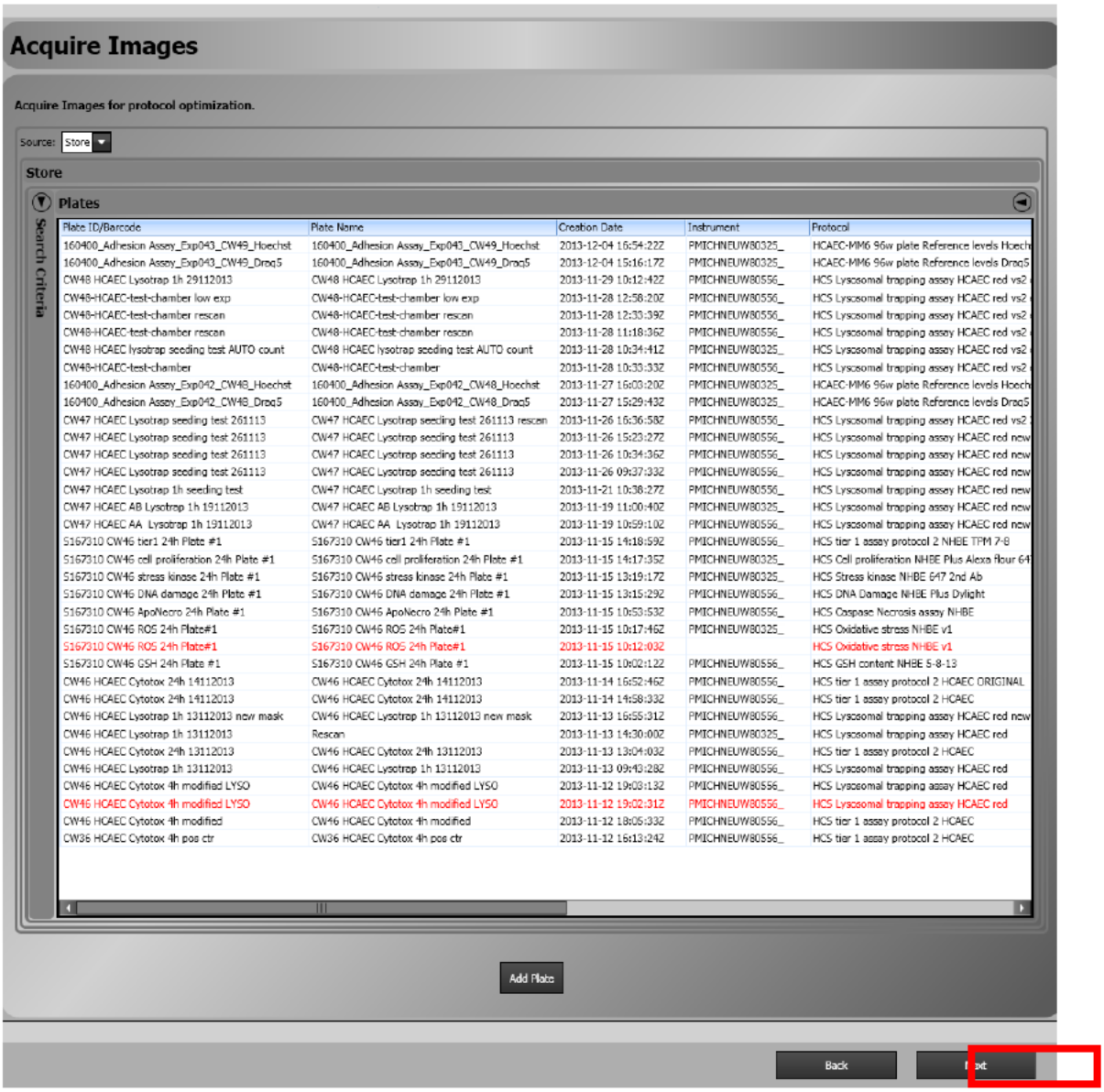




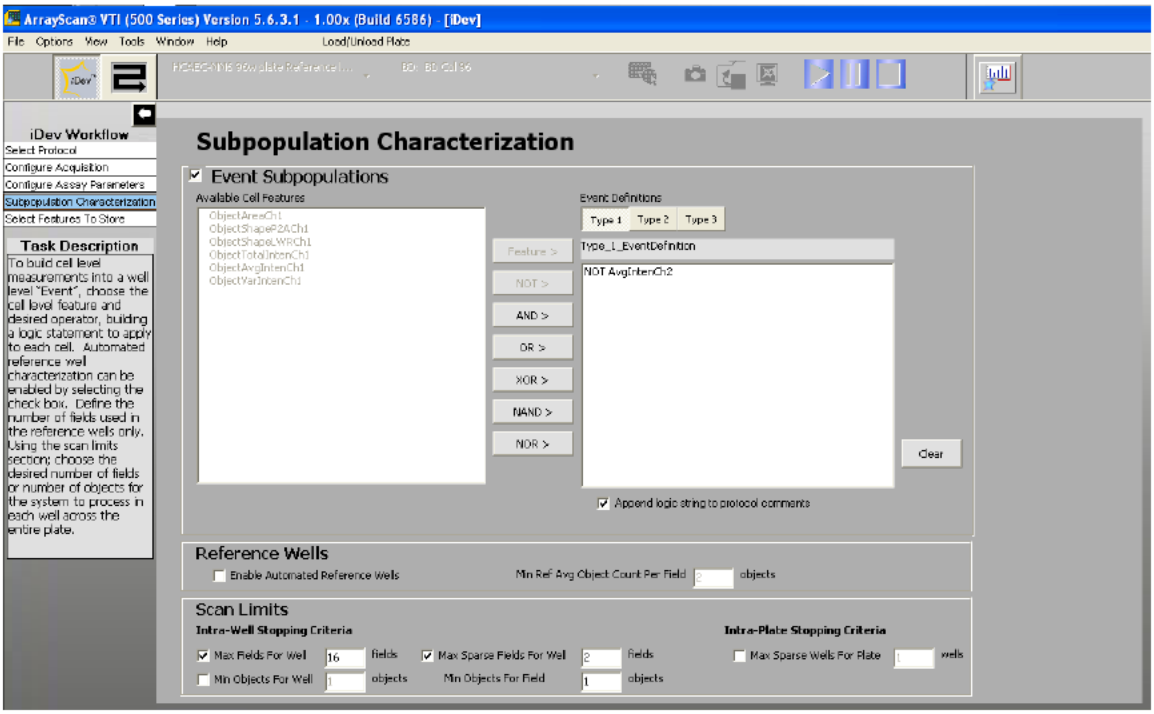
- Select the appropriate type of plate:
  - Click on “Change”, “BD” and “BD Col 96”
  - Click on “Next” at the bottom of the page



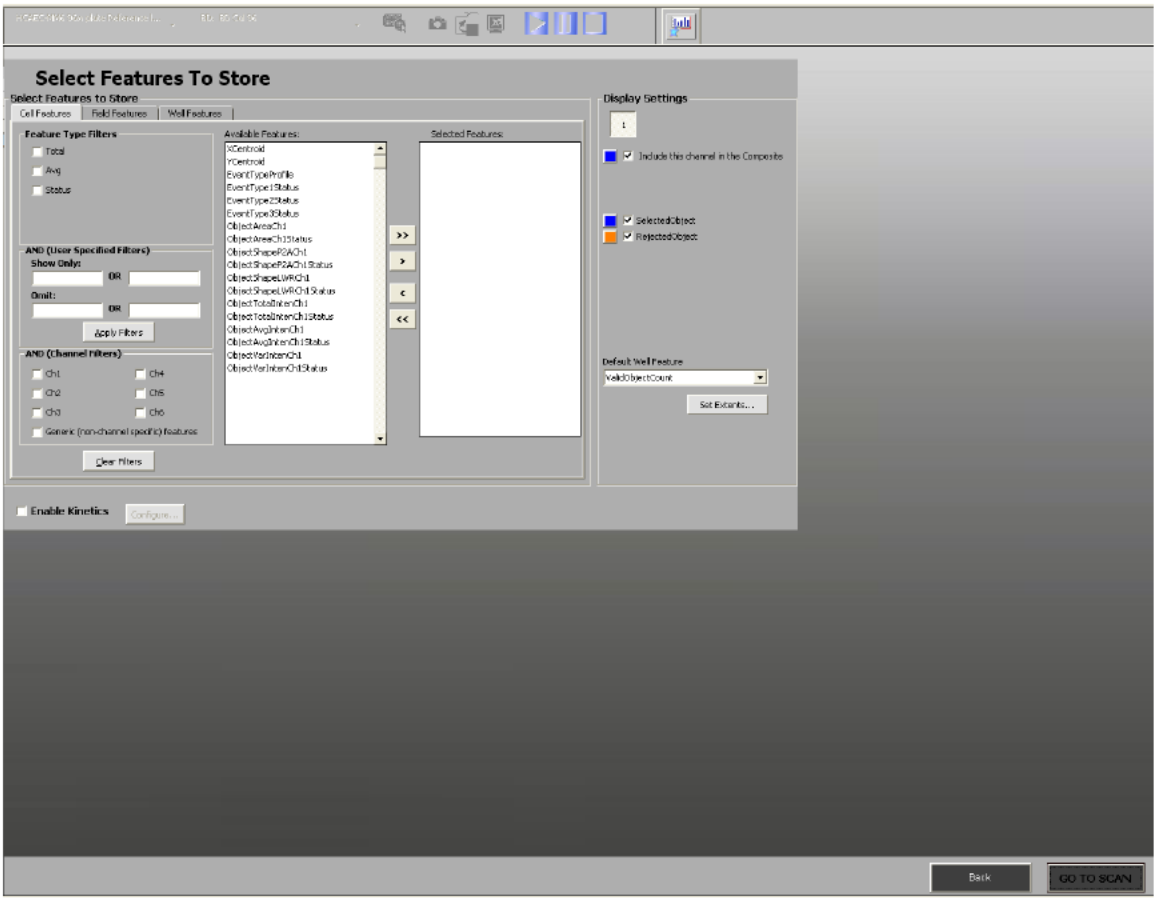
- Configure assay parameter / Acquire image
  - Click on “Next” at the bottom of the page

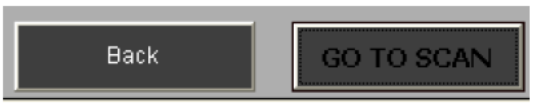


- Subpopulation characterization
  - Tick “Max fields for well” and type 16 fields
  - Tick “Max sparse fields for well” and type 2 fields
  - Click on “Next” at the bottom of the page

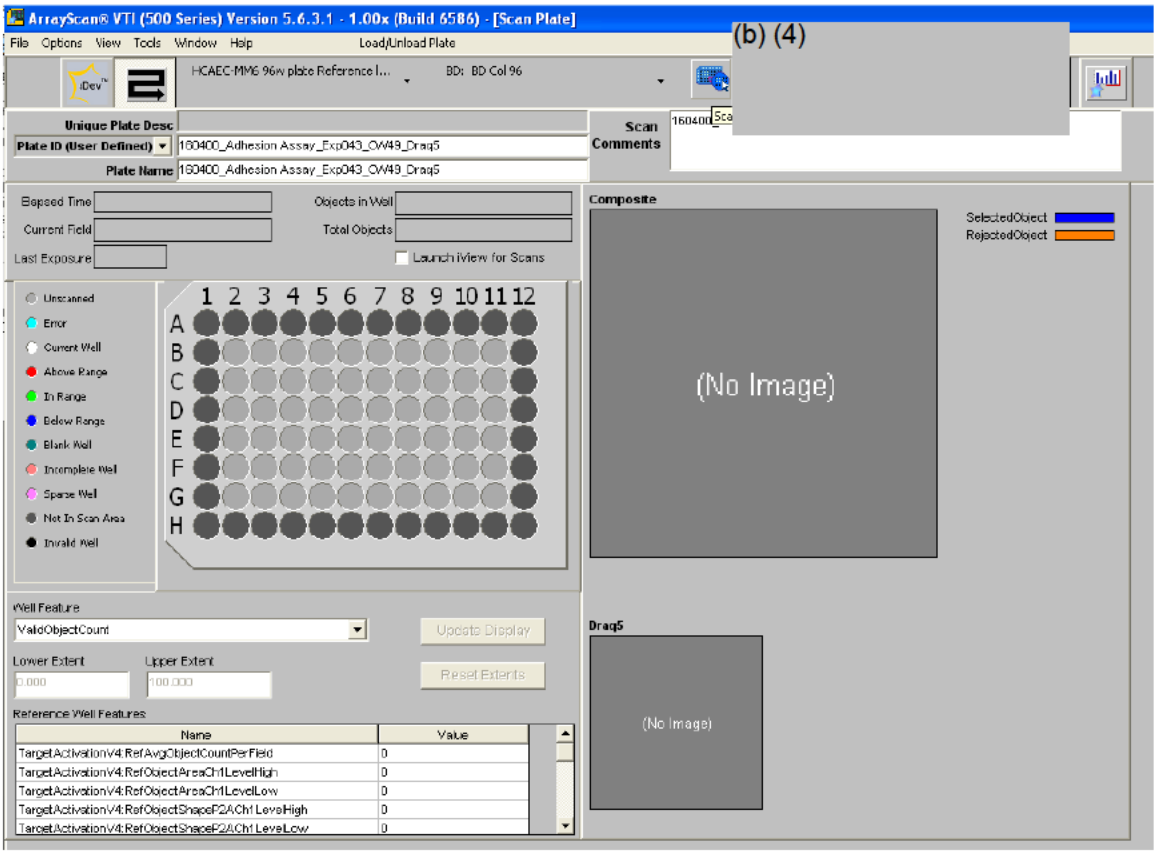


- Select features to store
  - Click on “Go to scan” at the bottom of the page





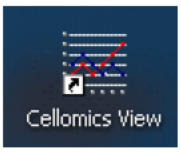
- Enter the plate name and select the wells to be scanned
  - In “Plate ID”, “Plate name” and “Scan comments”, type the information regarding pour assay: Project number, project name, experiment number, calendar week, channel used (e.g. ***160400_Adhesion Assay_Exp043_CW49_Draq5***)
  - To select the wells to be read, click on “Scan area selection”
  - Click on “Start” to start the reading of the plate

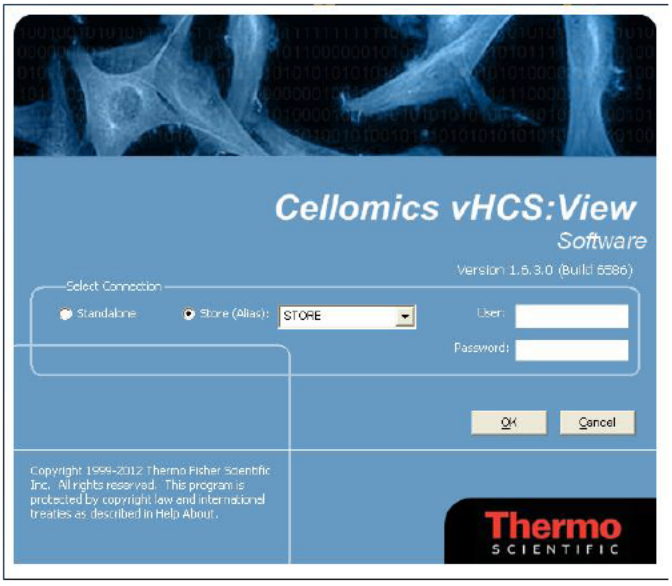


Once the plate is read, repeat the procedure using the Hoechst channel to count the HCAEC cells

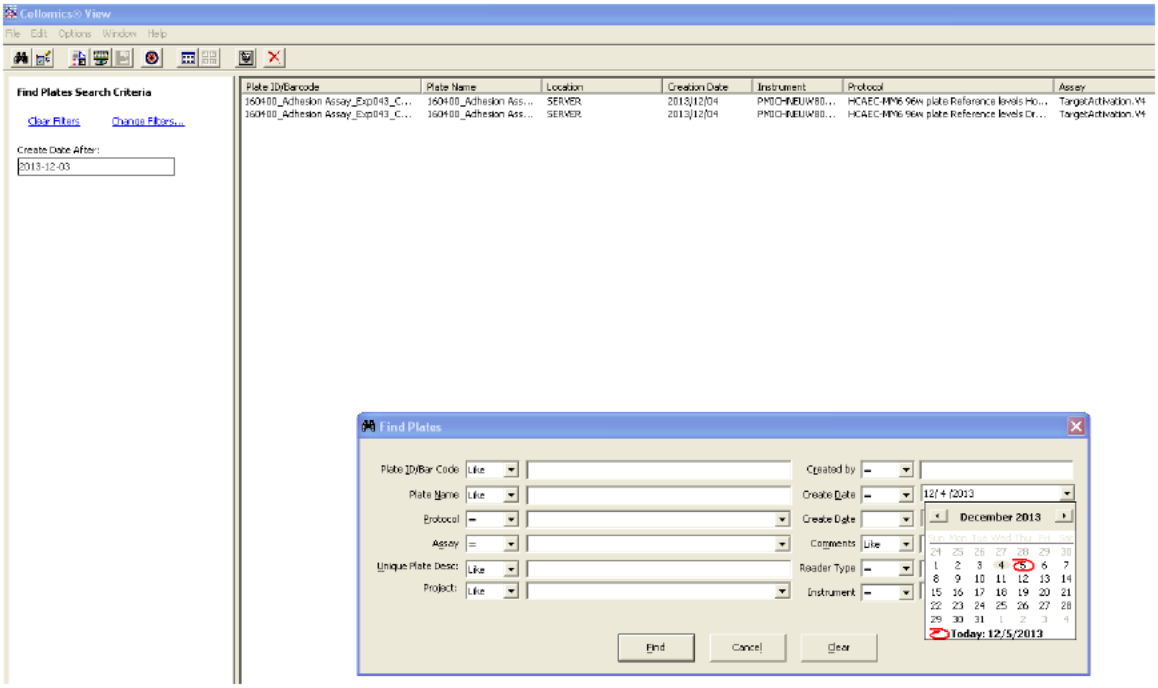
In "Target activation", choose the protocol "HCAEC-MM6 96w plate Reference levels Draq5"

- To retrieve raw data
  - Open iView
  - Login yourself:
    - User: your own PMI login
    - Password: (b) (4)

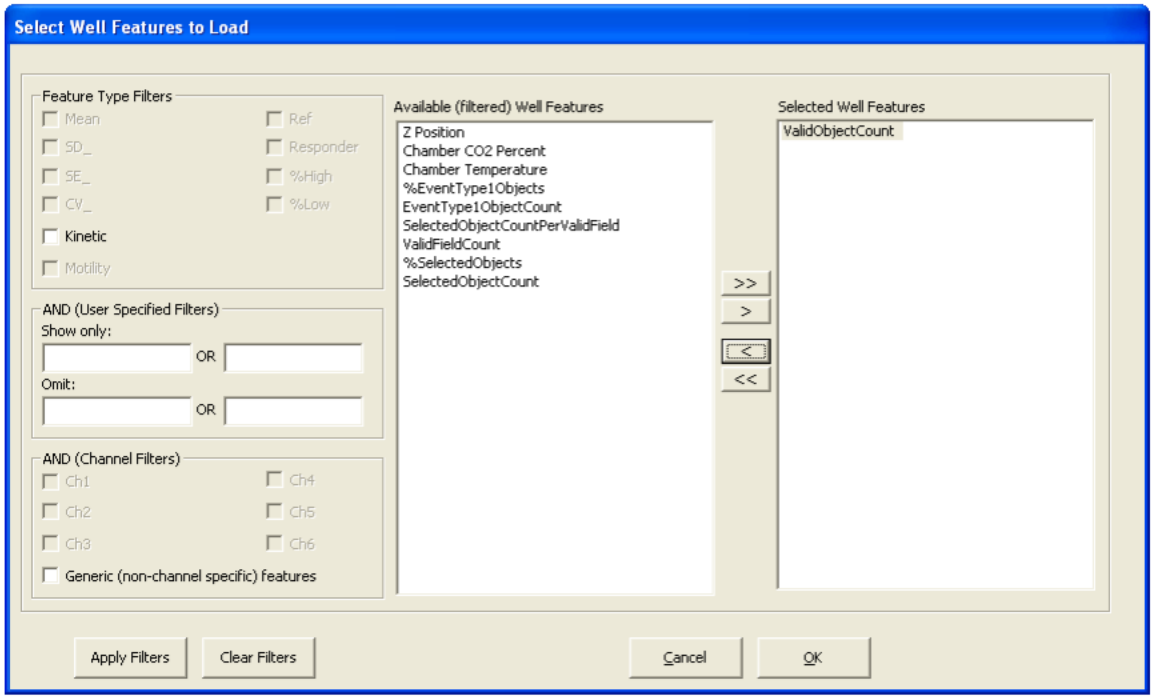




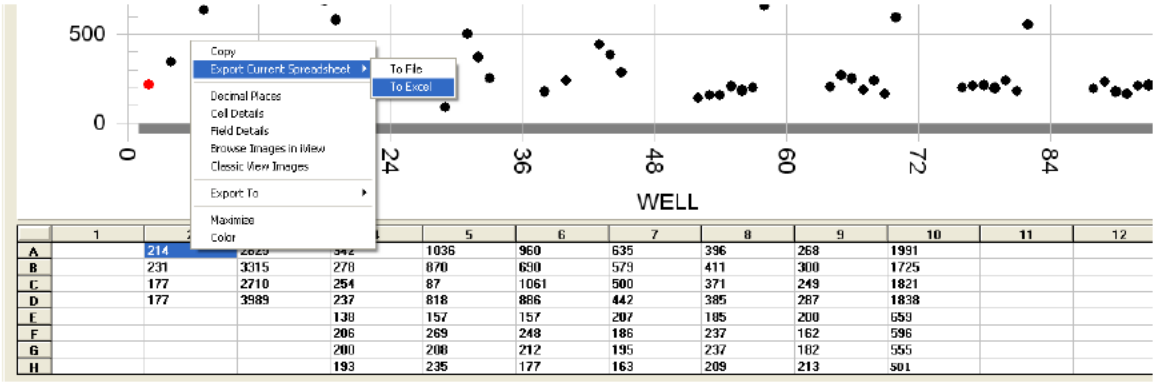
- Select your plate
  - Click on "Change filters" and find your plate with the date of the scan



- Select well features to load
  - Choose "Valid Object Count"



- Export your data to Excel
  - Right click on the raw data table
  - Select "Export Current Spreadsheet" then "To Excel"



	1	2	3	4	5	6	7	8	9	10	11	12
A		214	2829	342	1036	960	635	396	268	1991		
B		231	3315	278	870	690	579	411	300	1725		
C		177	2710	254	87	1061	500	371	249	1821		
D		177	3989	237	818	886	442	385	287	1838		
E				138	157	157	207	185	200	659		
F				206	269	248	186	237	162	596		
G				200	208	212	195	237	182	555		
H				193	235	177	163	209	213	501		

Calculate the adhesion rate in every well: (Draq5 value/Hoechst value) X 100

Calculate the average and the standard deviation of the replicates

Product references

PRODUCTS	SUPPLIER	CATALOG REF.
Hoechst bisBenzimide H 33342 trihydrochloride for fluorescence, ≥97.0%	Sigma	14533 – 100mg
TNF-α TNF-α Recombinant Human	Invitrogen	PHC3015 – 10µg
PBS Dulbecco's Phosphate Buffered Saline - Without MgCl2 and CaCl2	Sigma	D8537-6X500 mL
DMSO Dimethyl Sulfoxide	Sigma	D8418-100 mL
LPS Lipopolysaccharides from Escherichia coli 0111:B4	Sigma	L2630
ELISA – Human TNFα Human TNF-alpha DuoSet	R&D Systems	DY210
ELISA – Substrate Reagents Substrate Reagent Pack (8 vials Color A, 8 vials Color B)	R&D Systems	DY999
ELISA – Stop solution Stop Solution 2N Sulfuric Acid (15 x 6 mL)	R&D Systems	DY994
ELISA – Wash buffer Quantikine Wash Buffer 1	R&D Systems	WA126
ELISA – Reagent diluent Reagent Diluent Concentrate 2 (10X, 5 x 21 mL)	R&D Systems	DY995
ELISA – 96w plate Corning® 1 x 8 Stripwell™ 96 well plates	Sigma-Aldrich	CLS2592-25EA
ELISA – Sealing tape Corning® microplate sealing tape	Sigma-Aldrich	CLS3095-100EA

MM6 cells Acute monocytic leukemia	Ziegler-Heitbrock	s. DSMZ ACC 124
RPMI 1640 (for MM6 media)	Sigma	R0883
OPI Media Supplement (for MM6 media)	Sigma	O5003
FBS low LPS (for MM6 media)	PAA	A15-102
NEAA (100x) (for MM6 media)	PAA	M11-003
L-Glutamine (200mM) (for MM6 and HUVEC media)	PAA	M11-004
Penicillin/Streptomycin (100x) (for MM6 and HUVEC media)	Ruwag	P11-010
Human Coronary Artery Endothelial Cells	Vitaris	HCAEC lot 2121001.3
Growth medium: Endothelial Cell Growth Medium MV2 (Ready-to-use)	Vitaris	C-22022
Starvation medium: Endothelial Cell Growth Medium MV2 Kit	Vitaris	C-22121
BD BioCoat™ Collagen I, 175cm2	VWR	734-0292
BD Biocoat™ Collagen I coated, 96 well black/clear plates	Milian	356649
Cell culture plates for suspension cells 24-well	Sarstedt	83.1836.500
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (G7891)	Promega	G7891
96-Well Black Clear-Bottom Plates	Corning	3603
Formaldehyde solution	Sigma	F1635-25 mL

Signatures

This document has been read and approved by:

Name	Function	Justification	Signature, Date
Stefan Frentzel	Cellular Lab Manager	Signed as Peer Reviewer	27. Feb 2014 S. Frentzel
Alexandra Laurent	Laboratory Technician	Signed as Experiment Leader	04 March 2014
Carine Poussin	Study Director	Signed as Approver/Author	26 Feb. 2014
Hector De Leon	WP Leader	Signed as Approver	Not available
Carole Mathis	Project Leader	Signed as Approver	Not available
Julia Hoeng	Program Manager	Signed as Approver/Owner	27. Feb. 2014