INTRODUCTION

AIM 3D Cell Culture Chips feature a design that allows the application of shear flow or interstitial flow or the combination of them in the chips. With AIM Luer Connectors, syringes or flow systems with luer fittings can be connected to the chips easily. This modular expansion capability of the chips further enhances their functionality especially in terms of precise flow control. A stable concentration gradient across the hydrogel can be set up in AIM chips through the application of shear flow. This protocol covers the calculations and techniques for the application of flow in the chips.

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INTERSTITIAL FLOW ⏱ TIMING Variable

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- Cell-seeded AIM 3D Cell Culture Chips
- Syringe barrels (Becton Dickinson, Cat. No. 309659) or any other 1ml/2 ml syringes with luer tip styles
- C-flex tubing (Cole Parmer, Cat. No. 06422-01)
- Male luer lock adaptors with 1/16" barb end (Cole Parmer, Cat. No. 45518-07)
- 18 gauge blunt end needles (Nordson EFD, Cat. No. 7018122)

Please refer to the Connector Handling Protocol for the techniques (e.g. handling connectors and AIM Inlet Seals) to prepare chips for the following flow studies

Calculations before Experiments

i. You can use Darcy's equation to approximate the flow of fluid through a porous hydrogel:

$$Q = -\frac{kA}{\mu}\frac{\Delta P}{l}$$

Reminder *Darcy equation is used based on the assumptions that the cells do not cover the hydrogel interface and the fluid does not pass through the hydrogel inlets.*

Table 1 Definition of symbols used in Darcy's equation

Symbol	Definition	Value
Q	Fluid volume passing through hydrogel over time	Measured by users
k	Permeability of hydrogel	Estimated by using Darcy's law
A	Cross-sectional area	2.6×10^{-6} m^2
μ	Fluid viscosity	0.00078 Pa.s (DMEM at 37 °C)
ΔP	Pressure drop from one media channel to the other media channel	Set up by users
l	Length over which pressure drop takes place	0.0013m

ii. As the permeability of hydrogel, k varies with the type and concentration of hydrogel and also the conditions of gelation, you need to estimate its value for your particular application by using Darcy's equation. Once the value of k is determined for a particular application, you can continue to use it as long as the conditions of the hydrogel are the same

iii. To measure Q, which is the fluid volume passing through a hydrogel over time and to estimate the pressure drop from one media channel to the other media channel, we recommend the following two methods:

Method 1: Attach auxiliary reservoirs to the connectors and apply different volumes of fluid into the reservoirs to generate a pressure drop between media channels. We recommend using 1ml luer slip syringe barrels as auxiliary reservoirs.

a. Connect AIM Luer Connectors to a hydrogel-filled chip.

b. Pre-prime the connectors with 60 µl of medium then connect syringe barrels to the connectors.

Reminder *By using tubing, you may connect AIM chips to auxiliary reservoirs with much larger surface areas where the pressure drop between two media channels can be maintained relatively constant over time [1]*

c. Fill 0.5ml of medium into any two syringe barrels that are connected to the same media channel. And then fill 0.1 ml of medium into the other two syringe barrels.

d. The initial volume difference between the two media channels is ΔV_0.

For i =1

$$\Delta V_{i-1} = \Delta V_0 = 2 \times (0.5 - 0.1) = 0.8\ ml$$

e. Measure the initial height difference of medium in the syringe barrels in millimetre, for example 30 mm H_20

For i =1

$$\Delta P_{i-1} = \Delta P_0 = -30\ mmH_2O = -30 \times 9.8Pa = -294\ Pa$$

f. Based on the graduation scale on syringe barrels, record the change of volume hourly for at least 4 hours or until the medium in the two channels equilibrate ($\Delta V_i = 0$).

g. Estimate the flow rate Q_i

$$Q_i = \frac{\Delta V_{i-1} - \Delta V_i}{t}$$

h. Convert all values into S.I. unit and plug them into Darcy's equation. You then can calculate k_i based on the estimated Q_i and ΔP_{i-1}. Take the average value of at least 3 k_is as a representative value for the permeability of the hydrogel.

Reminder *This method is easy to perform but it only gives a rough estimation of k because the pressure drop varies with time. You may omit the values you obtain from the first hour as the pressure drop decreases very rapidly in the initial phase, making the first hour's estimates inaccurate.*

Method 2: Attach auxiliary luer adaptors, tubing & syringe pumps to the connectors to control the pressure drop between media channels more precisely.

a. Connect AIM Luer Connectors to a hydrogel-filled chip.

b. Pre-prime the connectors with 105 µl of medium and then connect male luer lock adaptors to the connectors.

c. Pre-prime the tubing that is connected to a syringe pump with medium.

d. Attach the pre-primed tubing to the male luer lock adaptor and make sure no air is trapped along the flow path. Attach empty tubing to the opposite connected male luer lock adaptor (of the same media channel) and direct the tubing to a collection container. Repeat this step for the other media channel.

e. Set the flow rate in one media channel at $\dot{V}_1$ (this is **NOT** interstitial flow rate, do not mix it up with Q that you need to measure), which is higher than the flow rate $\dot{V}_2$ that you set in the other media channel.

f. Start the syringe pumps and run them for a time period, t, for example 1h.

g. Measure the volume of medium, $V_{_total}$, collected from the outlet of the media channel with a lower flow rate, $\dot{V}_2$. If direct measurement of volume is impossible due to small amount of medium that is available, you can weigh the collected medium and then use its density to approximate its volume.

h. Calculate the fluid volume passing through the hydrogel, $V_{_interstitial\ flow}$ and flow rate, Q based on the measured $V_{_total}$.

$$V_{_interstitial\ flow} = V_{_Total} - \left(\dot{V}_2 \times t\right)$$

$$Q = \frac{V_{_interstitial\ flow}}{t}$$

i. Fluid flow in a media channel creates a pressure drop along the media channel (this is **NOT** the pressure drop used in Darcy's equation) and you can approximate the pressure drop by using the following equation as shown by Fuerstman et al[2].

$$\Delta P_1 = \frac{a\mu\dot{V}_1 l}{WH^3} = 39.1\mu\dot{V}_1$$

The unit for $\dot{V}_1$ is in μl/min, for μ is in Pa.s and for ΔP_1 is in Pa.

Reminder *You need to have two syringe pumps or a single syringe pump that can control two flow rates independently.*

Table 2 Definition of symbols used in the equation as shown by Fuerstman et al.

Symbol	Definition	Value
ΔP	Pressure drop along the media channel	Estimated by using this equation
a	A dimensionless constant depends on aspect ratio of the media channel	17.5
$\dot{V}_i$	Fluid flow rate in media channels as driven by syringe pumps	Set by users
μ	Fluid viscosity	0.00078 Pa.s (DMEM at 37 °C)
l	Length of media channel	0.00105m
W	Width of media channel	0.0005m
H	Height of media channel	0.00025m

j. Calculate the pressure drop from one media channel to the other media channel.

$$\Delta P = \Delta P_2 - \Delta P_1$$

k. Convert all values into S.I. unit and plug them into Darcy's equation. You then can calculate k based on the measured Q and estimated ΔP.

iv. Other than the two methods suggested here, you can also use a bead tracer method to measure the flow velocity directly by monitoring the fluorescent beads under flow condition. This method is accurate but it is not recommended here due to its complexity. You can refer to studies done by Polacheck et al and Vickerman et al for details [1, 3].

v. For applications that only require short term interstitial flow, the media ports can serve as reservoirs. You can estimate the pressure drop as following:

a. The initial volume difference between two media channels is ΔV_0, such as 40 μl.

b. Pressure drop can be estimated by dividing the ΔV_0 by the media port area, $A_{_media\ port}$ to get the height difference. $A_{_media\ port} = 19.6\ mm^2$

$$\Delta H = \frac{\Delta V_0}{2 * A_{media\ port}} \approx 1.01mm$$

$$\Delta P \approx 1.01 mm H_2 0 \approx 9.98\ Pa$$

Reminder *The change in volume is difficult to measure when the media ports are used as reservoirs. You should calculate permeability, k by using the previous two methods and then use k to estimate the flow rate in this case.*

Application of Interstitial Flow

1. You may refer to Table 3 for a list of publications that use interstitial flow in various applications.

Table 3 List of publications that use interstitial flow in various applications

Applications		References
Vascular function	Flow studies in a 3D capillary bed	[4]
	Angiogenesis in a 3D epithelial co-culture	[5]
	Effects of mesenchymal stem cells on angiogenesis	[6]
	Mechanism of a flow-gated angiogenesis switch	[3]
Cancer	Influence of interstitial flow on the direction of tumor cell migration	[1]
	Mechanotransduction of fluid stresses governs 3D cell migration	[7]
	Effects of anti-migratory drug and interstitial flow on tumor cell migration	[8]

2. **Option 1**: Attach auxiliary reservoirs to the connectors to generate pressure drop between media channels. We recommend using 1ml luer slip syringe barrels as auxiliary reservoirs (both luer slip and luer lock syringes can fit tightly with the connectors but two luer lock syringes are harder to be fitted adjacently due to the obstruction caused by the oversize flanges while screwing down).

    a. Remove medium from the connectors, if necessary.

    b. Add 60 µl of medium into each connector.

! Critical *If an insufficient amount of medium is added, an air gap will form after a syringe barrel is attached, thus preventing the medium from flowing into media channels.*

! Critical *Ensure that no air is trapped along the flow path. If fluid levels in two connected connectors (to the same media channel) do not equilibrate, apply negative or positive pressure by using a micropipette at the connectors to remove the trapped air.*

    c. Ensure the syringe barrels are sterile. Attach a 1ml syringe barrel (with plunger removed) into each connector. Wipe off spillage of medium, if any, from the connectors immediately. This is to avoid cross-contamination and also to prevent contact with AIM Inlet Seals.

Reminder *If larger reservoir is needed, 2ml syringe barrel may be used. Any syringe barrel larger than 2ml may not fit.*

d. Add 1 ml of medium each into the two syringe barrels that are attached to the same media channel that requires higher pressure head. Repeat this for the other two syringe barrels with lesser medium. The hydrostatic pressure difference between the two media channels generates interstitial flow across the gel region.

Reminder *The volume of medium added into syringe barrels can be varied to generate different flow rates depending on your specific application.*

Reminder *The interstitial flow rate decreases slowly over time when the volumes of medium equilibrate.*

Reminder *The time for the medium in both channels to equilibrate depends on the type of hydrogel and the initial volume difference.*

e. Replace culture medium daily (until the end of experiment) to reset the interstitial flow.

3. **Option 2**: Attach auxiliary luer adaptors, tubing & syringe pumps to the connectors to control the pressure drop between media channels more precisely. We recommend using male luer lock adaptors with 1/16″ ID low profile barb and biocompatible tubing. Tubing with sizes ranging from 1/16″ (for incompressible tubing) to 1/32″ ID (for compressible tubing) may be used. The corresponding needle sizes are 15 and 18 gauge respectively and we recommend using blunt end needles, not only to avoid injury but also to ensure the tubing is not punctured.

a. Ensure each connector has about 105 µl of medium.

! Critical *If too much medium is added, there is a risk of spillage while connecting male luer lock adaptors. If insufficient amount of medium is added, an air gap will form at the tip of the male luer lock adaptors, thus preventing the medium from flowing into media channels.*

b. Ensure the male luer lock adaptors, tubing and needles are sterile. Connect the male luer lock adaptors to the connectors. Medium should emerge from the barb end of the adaptors and form spherical caps.

! Critical *Connect the male luer lock adaptors slowly and carefully to avoid spillage.*

c. Pre-prime the tubing that is attached to a syringe through a blunt end needle with medium.

d. Attach the pre-primed tubing to the male luer lock adaptor and make sure no air is trapped along the flow path. Attach empty tubing to the opposite connected male luer lock adaptor (of the same media channel) and direct the tubing to a waste reservoir. Repeat this step for the other media channel.

e. In order to create an interstitial flow across the collagen, the flow rate of medium in one media channel has to be set higher than the other.

f. Keep the chips in an incubator. Depending on your syringe pumps and incubator set-up, the connected tubing can go through the dedicated hole on the incubator, through the incubator door, or be contained within the incubator if it is connected to a pocket size syringe pump system.

SHEAR FLOW ⏱ TIMING Variable

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- Cell-seeded AIM 3D Cell Culture Chips
- Syringe barrels (Becton Dickinson, Cat. No. 309659) or any other 1ml/2 ml syringes with luer tip styles
- C-flex tubing (Cole Parmer, Cat. No. 06422-01)
- Male luer lock adaptors with 1/16" barb end (Cole Parmer, Cat. No. 45518-07)
- 18 gauge blunt end needles (Nordson EFD, Cat. No. 7018122)

Calculations before Experiments

i. Shear stress, τ is directly proportional to dynamic viscosity, μ and flow rate, Q

$$\tau \propto \mu \times Q$$

$$\tau = \mu \times K \times Q$$

ii. The constant, K is dependent on the geometry of media channels and the units used.

Table 4 Formulas to calculate shear stress in the channels

International System, SI unit		Centimetre-gram-second, CGS unit	
τ	Pa	τ	dyne/cm^2
μ	Pa.s	μ	dyne.s/cm^2
Q	μl/min*	Q	μl/min*
	$\boldsymbol{\tau = \mu \times 3.11 \times Q}$		$\boldsymbol{\tau = \mu \times 3.11 \times Q}$

* μl/min is neither a SI unit nor CGS unit but is used here for convenience, due to the small flow rate that users will typically use

iii. For typical cell culture media e.g. DMEM or F12, you may use the lookup table as a quick reference to determine the required flow rate for a specific shear stress. For detailed information on how the shear stress value is derived for a rectangular channel, please refer to Son 2007 [9].

Table 5 Lookup table for shear stress levels and the corresponding flow rates

τ [Pa]	Q [μl/min]	τ [dyne/cm^2]	Q [μl/min]
0.001	0.412	0.01	0.412
0.005	2.06	0.05	2.06
0.01	4.12	0.1	4.12
0.05	20.6	0.5	20.6
0.1	41.2	1.0	41.2
0.5	206	5	206
1	412	10	412
5	2060	50	2060
10	4120	100	4120

These values are calculated based on the dynamic fluid viscosity of DMEM at 37 °C which is μ = 0.00078 Pa.s = 0.0078 dyne.s/cm^2 [10]

Reminder *The viscosity of media changes with temperature and pressure. Additives for media (e.g. macromolecules) may change the viscosity of media too.*

Application of Shear Flow

4. Attach auxiliary luer adaptors, tubing & syringe pumps to the connectors to control the flow rates more precisely. We recommend using male luer lock adaptors with 1/16″ ID low profile barb and biocompatible tubing. Tubing with sizes ranging from 1/16″ (for incompressible tubing) to 1/32″ ID (for compressible tubing) may be used. The corresponding needle sizes are 15 and 18 gauge respectively and we recommend using blunt end needles, not only to avoid injury but also to ensure the tubing is not punctured.

    a. Ensure each connector has about 105 µl of medium.

    ! Critical *If too much medium is added, there is a risk of spillage while connecting male luer lock adaptors. If insufficient amount of medium is added, an air gap will form at the tip of the male luer lock adaptors, thus preventing the medium from flowing into media channels.*

    b. Ensure the male luer lock adaptors, tubing and needles are sterile. Connect the male luer lock adaptors to the connectors. Medium should emerge from the barb end of the adaptors and form spherical caps.

    ! Critical *Connect the male luer lock adaptors slowly and carefully to avoid spillage.*

    c. Pre-prime the tubing, which is attached to a syringe through a blunt end needle, with medium.

    d. Attach the pre-primed tubing to the male luer lock adaptor and make sure no air is trapped along the flow path. Attach empty tubing to the opposite connected male luer lock adaptor (to the same media channel) and direct the tubing to a waste reservoir. Repeat this step for the other channel.

    e. Set the required flow rate by using syringe pumps.

    Reminder *The flow rates in both channels have to be the same to prevent any pressure difference across the collagen gel region.*

    Reminder *Other than using two syringe pumps, you may also utilize a Y-connector to split the flow from a single syringe pump before directing the medium to the media channels. Make sure the flow paths are identical after splitting. The effective flow rate in a single media channel will be halved hence the formulas shown earlier need to be accounted for this.*

    f. Keep the chips in an incubator. Depending on your syringe pumps and incubator set-up, the connected tubing can go through the dedicated hole on the incubator, through the incubator door, or be contained within the incubator if it is connected to a pocket size syringe pump system.

    Reminder *We do not recommend applying shear flow with syringe barrels as the water levels equilibrate very quickly within the same media channel.*

MAINTAINING CONCENTRATION GRADIENTS ⏱ TIMING Variable

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3202) enriched with chemicals/proteins of interest at two different concentrations, e.g. medium enriched with 20 ng/ml of vascular endothelial growth factor (VEGF) vs medium without enrichment

Others

- Cell-seeded AIM 3D Cell Culture Chips
- Syringe barrels (Becton Dickinson, Cat. No. 309659) or any other 1ml/2 ml syringes with luer tip styles
- C-flex tubing (Cole Parmer, Cat. No. 06422-01)
- Male luer lock adaptors with 1/16" barb end (Cole Parmer, Cat. No. 45518-07)
- 18 gauge blunt end needles (Nordson EFD, Cat. No. 7018122)

Calculations before Experiments

i. Péclet number, Pe is a dimensionless number that represents the relative importance of convection to diffusion in fluid transport phenomena. When the Pe is >>1, convective transfer is more significant than diffusive transfer and the *vice versa* holds when the Pe is <<1. In order to make sure the solute species (eg. VEGF) get replaced timely to maintain a stable concentration gradient, Pe should be >>1. Pe is the product of fluid velocity, u and characteristic length, L that is divided by diffusion coefficient, D.

$$Pe = \frac{uL}{D} \gg 1$$

ii. L can be approximated as the channel width, w in a microfluidic channel [11]. We also know that the cross sectional area, A is the product of channel width, w and channel height, H. Rearrange the equation by substituting fluid velocity, u with volumetric flow rate, Q and cross sectional area, A and we get the following expression.

$$Pe = \frac{Q \times w}{A \times D} \gg 1$$

$$\frac{Q \times w}{(w \times H) \times D} \gg 1$$

$$\mathrm{Q} \gg HD$$

$$\mathrm{Q} \gg 2.5 \times 10^{-4} D$$

Where D (m^2/s) is the diffusion coefficient of solute in the medium.

iii. As demonstrated by Zervantonakis et al. [11], Pe should be equal to or greater than 10 in order to yield a stable concentration gradient across the hydrogel.

$$\mathrm{Q} \geq 2.5 \times 10^{-3} D$$

iv. Once the diffusion coefficient for a particular protein/chemical is known, you can determine the minimum flow rate Q to achieve a constant concentration gradient across hydrogel. The following lookup table is included for your ease of reference.

Table 6 Lookup table for flow rates to maintain stable concentration gradient across hydrogel and the corresponding diffusion coefficients

D **[m^2/s]**	**Q [m^3/s]**	**Q [$\mu l/min$]**
1×10^{-11}	2.5×10^{-15}	0.0015
2×10^{-11}	5.0×10^{-15}	0.003
3×10^{-11}	7.5×10^{-15}	0.0045
4×10^{-11}	1.0×10^{-14}	0.006
5×10^{-11}	1.3×10^{-14}	0.0075
6×10^{-11}	1.5×10^{-14}	0.009
7×10^{-11}	1.8×10^{-14}	0.0105
8×10^{-11}	2.0×10^{-14}	0.012
9×10^{-11}	2.3×10^{-14}	0.0135
10×10^{-11}	2.5×10^{-14}	0.015

Reminder *The minimum required flow rate to maintain a stable concentration gradient in AIM chips is usually very low and its corresponding shear stress is often negligible.*

Application of Shear Flow to Maintain Concentration Gradients

5. Attach auxiliary luer adaptors, tubing & syringe pumps to the connectors to control the flow rates more precisely. We recommend using male luer lock adaptors with 1/16″ ID low profile barb and biocompatible tubing. Tubing with sizes ranging from 1/16″ (for incompressible tubing) to 1/32″ ID (for compressible tubing) may be used. The corresponding needle sizes are 15 and 18 gauge respectively and we recommend using blunt end needles, not only to avoid injury but also to ensure the tubing is not punctured.

    g. Ensure each connector has about 105 µl of medium.

    ! Critical *If too much medium is added, there is a risk of spillage while connecting male luer lock adaptors. If insufficient amount of medium is added, an air gap will form at the tip of the male luer lock adaptors, thus preventing the medium from flowing into media channels.*

    h. Ensure the male luer lock adaptors, tubing and needles are sterile. Connect the male luer lock adaptors to the connectors. Medium should emerge from the barb end of the adaptors and form spherical caps.

    ! Critical *Connect the male luer lock adaptors slowly and carefully to avoid spillage.*

    i. Pre-prime the tubing, which is attached to a syringe through a blunt end needle, with growth factor-enriched medium.

    j. Attach the pre-primed tubing to the male luer lock adaptor and make sure no air is trapped along the flow path. Attach empty tubing to the opposite connected male luer lock adaptor (to the same media channel) and direct the tubing to a waste reservoir. Repeat this step for the other channel with another growth factor-enriched medium.

    k. Set the required flow rate by using syringe pumps. The flow rates in both channels have to be the same to prevent any pressure difference across the collagen gel region.

    Reminder *Other than using two syringe pumps, you may also utilize a Y-connector to join the flow downstream and then connect the Y-connector to a single withdrawal pump. Syringe barrels that contain different growth factor-enriched media can be set up as upstream reservoirs. Make sure the flow paths are identical.*

    l. Keep the chips in an incubator. Depending on your syringe pumps and incubator set-up, the connected tubing can go through the dedicated hole on the incubator, through the incubator door, or be contained within the incubator if it is connected to a pocket size syringe pump system.

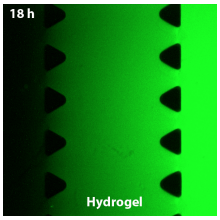

Figure 1 A fluorescent image showing a stable concentration gradient. This was taken 18 h after the infusion of dye-enriched medium into the right media channel and dye-free medium into the left media channel. The flow rate (0.08 µl/min) was 10 × higher than the minimum flow rate (**Table 6**) for the corresponding diffusivity of the dye.

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