



# Assessment of a New Cell Culture Perfusion Apparatus for *In Vitro* Chronic Toxicity Testing Part 1: Technical Description

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## Summary

*In vitro* models for chronic toxicity, defined as a recurring exposure to compounds over a prolonged period of time, are still underrepresented in drug evaluation processes. The classical approach to cell culture is not readily suitable to long term repetitive applications. Therefore, we assessed the use of a commercially available perfusion cell culture apparatus in its applicability to chronic renal toxicity testing and describe the technical aspects of adopting the perfusion cell culture system to our purposes. It was apparent that there is a subtle dynamic difference between human renal proximal tubular cells cultured under perfusion and static conditions as illustrated by the accumulation of lactate dehydrogenase (LDH) and the secondary metabolism of resazurin to hydroresorufin, which occurred only under static conditions. The major achievement was the standardisation of the handling of this system with regard to cell cultivation, pH regulation, temperature regulation, and reproducibility of common toxicity endpoints.

**Zusammenfassung:** Evaluierung eines neuen Zellkultur-Perfusionssystems für chronische *in vitro* Toxizitätstests. Teil 1: Technische Beschreibung

*In vitro* Modelle für chronische Toxizität, definiert als die wiederholte Gabe einer Substanz über einen längeren Zeitraum, sind noch immer unterrepräsentiert. Die konventionelle „statische“ Zellkultur ist kein ausreichendes Modell für Untersuchungen der chronischen Toxizität. Aus diesem Grund wurde ein kommerziell erhältliches Zellkultur-Perfusionssystem auf dessen Anwendbarkeit für die Prüfung chronischer Nephrotoxizität hin untersucht. In der vorliegenden Arbeit werden die technischen Aspekte und Modifikationen am Perfusionssystem beschrieben, die durchgeführt wurden, um das System für die spezifischen Anforderungen nutzbar zu machen. Beim Vergleich beider Modelle unter Verwendung humaner renaler Tubulusepithelzellen kam es unter statischen, nicht jedoch unter Perfusionsbedingungen, zur Akkumulation der Laktatdehydrogenase und zum sekundären Metabolismus von Resazurin zu Hydroresorufin. Zusammenfassend kann gesagt werden, dass eine Standardisierung in Bezug auf Zellkultivierung, pH-Regulation, Temperaturregulation und Reproduzierbarkeit von Endpunkten der Toxizität erreicht werden konnte.

**Keywords:** perfusion, chronic, toxicity, cell culture

## 1 Introduction

Many of the compounds we are in daily contact with have the potential to cause serious health problems. Whether these compounds are environmental pollutants, industrial chemicals or therapeutic agents, the deleterious health effects may take years or decades to develop. The kidney is one of the major target organs of

toxin-induced damage. It has been estimated that therapeutic agents cause 20% of all diagnosed end-stage renal disease (ESRD) and that chemicals and drugs may play a significant role in at least 50% of ESRD cases of unknown aetiology (Commission of the European Communities/International Program on Chemical Safety, 1989). Retrospective evaluation of the effects of chronic expo-

sure in human populations is confounded by the many different substances one individual may be exposed to at any given time. Animal models or *in vitro* systems are required to aid in the identification of potential toxins. Animal models themselves are problematic due to genetic differences (such as species differences in metabolising enzymes) and due to ethical concerns. However, the accumulation of a wealth of biochemical and cellular mechanisms together with advances in cell and tissue culture now permit the

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development and use of *in vitro* toxicity assays. The aim of the development of such *in vitro* tests is not only to refine, reduce and replace *in vivo* animal testing, but also to improve the relevance of data obtained for the safety evaluation for humans.

Strategies for renal toxicological studies range from whole organ to isolated nephron segments to individual cells (Pfaller and Gstraunthaler, 1998). For chronic nephrotoxicity testing, cell culture systems represent the most valid approach, mainly due to the possibility to carry out stable long-term toxic exposures on well-defined models without the complications of higher organ systems. Thus, one strategy to identify potential chronic nephrotoxins would be to further develop *in vitro* renal cell culture systems to allow for "chronic" application of toxins.

Chronic toxicity is defined as recurring (continuous or repeated) exposure to a low concentration of toxin over a prolonged period of time. The classical approach to renal cell culture is not readily applicable to "chronic" exposures. The constraints of conventional cell culture techniques make continuous exposure to toxins impractical if not impossible. An experiment in which a compound is applied once to the culture medium is analogous to an acute exposure. A high acute dose of toxin is often used as a predictive indication for low dose chronic exposure. A better prediction of chronic exposure would be the continual addition of a compound to the nutrient medium. To this end we have evaluated the applicability of a commercially available perfusion cell culture apparatus to toxicity studies. This project was conducted by 3 European laboratories as part of the IV<sup>th</sup> framework initiative, entitled "Development of a standardized *in vitro* methodology for hepatic and renal toxicity testing". The objectives of this project were: (i) to establish, characterise and optimise *in vitro* models for the assessment of toxicity, with particular emphasis on the perfusion technology (ii) to develop relevant and reproducible *in vitro* toxicity endpoints and set up standard operating procedures (iii) to exchange and prevalidate a selection of successful models.

The aim of this paper is to discuss the technical problems that were encountered with the perfusion cell culture system and to describe approaches taken to overcome these difficulties.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Alamar Blue was obtained from Accumed International Ltd. Human fibronectin and rat-tail collagen were obtained from Becton Dickinson. Foetal calf serum, L-alanyl-L-glutamine (glutamax), penicillin, streptomycin and glutamine were obtained from GibcoBRL. Methylene blue was obtained from Merck. All other reagents and chemicals were obtained from Sigma at the highest grade available. Reagents and chemicals used for cell culture were of tissue culture grade and had been tested for endotoxin.

### 2.2 Cell culture

The human proximal tubular cell line HK-2 (obtained from American Tissue Type Culture Collection) was maintained in DMEM Ham's F12 containing 5 mM glucose, 15 mM HEPES, 14 mM sodium bicarbonate, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-alanyl-L-glutamine (glutamax). Cells were grown to confluence in 75 cm<sup>2</sup> Falcon flasks and maintained at 37°C in a humidified atmosphere of 95% air, 5% CO<sub>2</sub>. Culture medium was changed every second or third day.

### 2.3 Microporous supports

Cells for perfusion and non-perfusion static controls were grown on either polycarbonate filters (13 mm, 1mm pore size) or aluminium oxide filters (13 mm diameter, 0.2 µm pore size) in filter carriers (Minucells and Minutissue Vertriebs GmbH, Germany). Filters were obtained from Whatman. Filters were coated with 2 µg/cm<sup>2</sup> human fibronectin in PBS for 1 h and were washed twice in medium prior to use. Cells were seeded onto coated filters at high density, approx. 4 x 10<sup>4</sup> cells/cm<sup>2</sup>, ensuring confluence to be reached within 24 h.

### 2.4 Preparation of cell homogenates

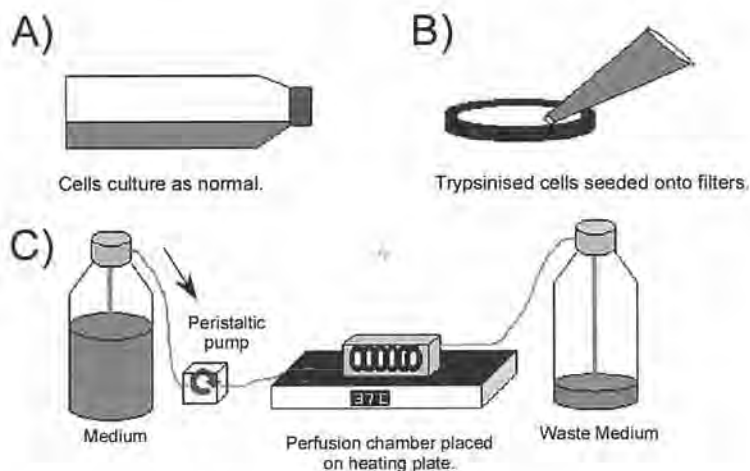
Cell monolayers on filters in filter carriers were transferred to 24 well plates and washed twice with PBS. The filters were then removed from the filter holders and washed a third time in cold PBS on ice. 100 µl of 4°C lysis buffer (0.1% (v/v) Triton X-100 in 50 mM Tris, pH 7.4) was added to each well of a 24 well plate containing 1 filter per well. The 24 well plates were frozen at -20°C until required. Cell contents were scraped from the filters and centrifuged at 20,000 x g for 10 min at 4°C. Supernatants were retained. Protein determination was carried out as described by Bradford using Biorad's Protein reagent, with BSA as a standard (Bradford, 1976).

### 2.5 Enzyme assays

Lactate dehydrogenase (LDH) activity was determined in cell homogenates and cell culture supernatants using a Boehringer Mannheim (Roche) LDH kit. Gamma-glutamyl transpeptidase (GGT) activity was determined in cell homogenates and cell culture supernatants using a miniaturised (96 well based) modification of the Sigma Diagnostics Assay No. 545.

### 2.6 Alamar Blue Assay

**End point Alamar Blue Assay:** Cells grown on filters were transferred to 24 well plates in filter carriers and washed 3 times in PBS. 650 µl of 10% Alamar Blue in completed cell culture medium (pre-warmed to 37°C) was added to each well of the 24 well plate, shaken gently and incubated in a 5% CO<sub>2</sub>, 37°C, humidified incubator for 2 h. 100 µl of cell supernatant was diluted with 2.9 ml de-ionised H<sub>2</sub>O (1 in 300 dilution of Alamar Blue) and read on a Perkin-Elmer LS-5 Spectrofluorometer with 560 nm excitation and 590 nm emission. The instrument was previously calibrated with 1/300 non-reduced Alamar Blue (to establish minimum reading) and 1/300 reduced Alamar Blue (to establish maximum reading). Each experimental replicate was measured in duplicate. **Continuous Alamar Blue monitoring:** Real time Alamar Blue determination was achieved by adding 0.2% v/v Alamar Blue to the cell culture. Samples were



**Fig. 1: Diagram of the cell culture perfusion system.**

(A) Cells are grown under standard laboratory conditions. (B) After trypsinisation cells are seeded onto filters in filter holders. (C) Once cells have attached to filters, they can be transferred to the perfusion chamber and perfusion is commenced.

drawn at different intervals and measured for fluorescence intensity.

## 2.7 Perfusion cell culture equipment and handling

### 2.7.1 Description of original perfusion system

The perfusion cell culture apparatus was obtained from Minucells and Minutissue Vertriebs GmbH, Germany and has been described in detail previously (Minuth et al., 1996). This apparatus consisted of filter holder rings (filter carriers), a perfusion culture chamber with the capacity to hold six filter carriers vertically, a reservoir and waste bottle with vented caps containing inlet and outlet holes, an 8-position peristaltic pump (IPC8, Ismatec, Germany), a heating plate (Storktronic, Germany), silicone connective tubing, silicone pump tubing and a variety of luer connectors (Fig. 1). Cells are seeded onto the filters in filter carriers. After cell adherence, the filter carriers are transferred to the perfusion chambers. Medium is pumped from the reservoir bottle through the silicone tubing via the peristaltic pump, then through the perfusion chamber and finally out into the waste bottle. The perfusion chamber is placed on the heating unit to maintain physiological temperature. The system is oxygenated by equilibration of the medium with external air via the gas-permeable silicone tubing.

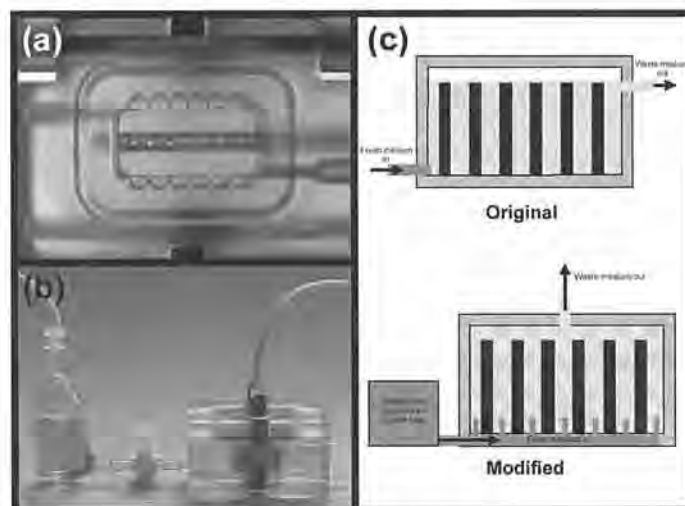
### 2.7.2 Use of perfusion system

Assembled lines (bottles, venting caps, tubing and chamber) were sterilised by autoclaving at 121°C, 1 bar of pressure for 30 min in autoclave bags with the chamber lid open. (Autoclaving at these settings was not recommended by manufacturers, but no deleterious effects to the equipment were noticed using this

method). Filter inserts containing adhered cells were placed into the chambers using sterile forceps and the chamber was closed. All connections were secured and lids tightened before removing the system from the sterile laminar flow. Pump tubing was connected to the pump and the closed chamber was placed on the heating unit (or in an enclosed incubator) under normal non-sterile laboratory conditions. The perfusion rate was set to 1 ml/h, for all experiments, as described previously (Minuth et al., 1997). After termination, experiment chambers were washed first in excess tap water and then in deionised water. Pump lines were cleaned by perfusion with deionised water at high speed.

### 2.7.3 Perfusion chamber design modifications

From the outset of this study, it was suspected that the perfusion chamber needed some modifications in order to improve the flow distribution between the filter holder inserts. The modification involved the drilling of a new inlet bore at the base of the chamber (37.4 mm in length, 4.25 mm diameter). Using a metal template, 7 holes of 0.8 mm diameter were made to connect the new inlet to the



**Fig. 2: Modification of the perfusion chamber.**

The perfusion chamber was drilled through the base to form a new inlet channel 37.4 mm in length and 4.25 mm diameter. Using a metal template 7 smaller inlets (0.8 mm diameter) were drilled from the inner chamber to the new channel (a). This adaptation allows cell culture medium to enter between the filter carriers. To prevent air bubbles entering the chamber and blocking the small inlets, a bubble trap was introduced in front of the chamber (b). A diagrammatic view of the differences between original and modified chambers is shown (c).



internal chamber, thus allowing a separate inlet between each individual sheet. To prevent air bubbles blocking the small inlet holes, a glass bubble trap was introduced before each chamber (Fig. 2 B, C).

### 2.7.4 Homogeneity of liquid distribution

To investigate whether the modifications applied improved the flow distribution within the chamber, a dye experiment was conducted. DMEM Hams F12 medium without phenol red was perfused at 1 ml/h through original and modified chambers containing 6 filter carriers with polycarbonate membranes at 37°C. At time 0, the colourless medium was replaced with medium containing 0.08 g/l methylene blue. Flow distribution was traced by photography at 10 min intervals using back lighting to aid visualisation.

### 2.7.5 Biochemical comparison between filter carrier positions

HK-2 cells, grown on fibronectin-coated polycarbonate filters, were maintained under identical perfusion conditions in 3 original and 3 modified chambers for 72 h. Hormonally defined DMEM Ham's F12 with 5 mM glucose was perfused through Pharmed tubing at 1 ml/h, with continuous bubbling with 5% CO<sub>2</sub>, 95% air. An endpoint Alamar Blue assay was performed on each filter. LDH activity and protein content was determined in cell homogenates. Data obtained was analysed for correlation with the position of the filter in the chamber and for variance.

### 2.7.6 Perfusion and static cell culture conditions

We aimed to keep every condition exactly identical in the static (non-perfusion) controls as well as in the perfusion experiments as far as possible. Thus all experiments were paired for cell population, filter batch, matrix coating and medium components. *Common conditions:* Cells were seeded onto fibronectin microporous filters in filter carriers in 10 cm diameter Petri dishes. Petri dishes were kept in a humidified 37°C, 5% CO<sub>2</sub>, 95% air incubator. When cell monolayers had reached confluence 1 to 2 days later, as evidenced by phase contrast mi-

croscopy, the filter carriers were randomly picked and transferred with sterile forceps to perfusion or static conditions. *Perfusion culture conditions:* Confluent cell monolayers in filter carriers were transferred to the modified perfusion chamber with 6 carriers per chamber. Cell culture medium from the reservoir bottle was pumped into the perfusion chamber via an Ismatec peristaltic pump at a rate of 1 ml/h. A glass bubble trap prevented gas trapped in the liquid from entering the chamber. The perfusion chambers were maintained at 36.5 to 37.0°C in an enclosed incubator (Stuart Scientific). Cell culture medium was bubbled slowly with a 5% CO<sub>2</sub>, 95% air mixture in the reservoir bottles, which were maintained at room temperature. To maintain CO<sub>2</sub> and oxygen saturation of the cell culture medium whilst in transit to the perfusion chambers, all tubing lines were CO<sub>2</sub>-impermeable (Pharmed). Effluent cell culture medium from the perfusion chambers was collected in sterile vented glass bottles. Sterile 3-way stopcocks (Baxter) were attached to the effluent line so that samples could be aseptically collected when required. Each filter in a perfusion chamber was considered a replicate for endpoint measurements. *Static culture conditions:* Confluent cell monolayers in filter carriers were transferred to new plastic Petri dishes (either 10 cm or 6 well dishes with 20 ml and 3 ml medium respectively) and maintained in a 37°C, humidified, 5% CO<sub>2</sub>, 95% air incubator for the duration of the experiment without medium replenishment. The distance of the cell monolayers from the liquid-air interface was not more than 2 mm in any of the static experimental set-ups. The same batch of complete medium was used for both static and perfusion conditions.

### 2.7.7 Monitoring of HK-2 cells under perfusion and static culture conditions

HK-2 cells were grown to confluence on collagen-coated aluminium oxide filters. At day 0, twelve microporous supports in filter carriers were transferred to two 10 cm dishes (six carriers per dish), each containing 20 ml medium, where they remained in a 37°C, 5% CO<sub>2</sub>, 95% air, humidified incubator for 7 days. Twelve

filter carriers were also maintained under perfusion conditions (in two perfusion systems) for 6 days. Medium used for both conditions was hormonally defined DMEM Ham's F12 containing 0.2% (v/v) Alamar Blue. 100 µl samples were taken from both static culture dishes every 24 h (pooled for assay). Medium was collected over a 1 h period from the perfusion line every 24 h. All samples collected were kept light-protected at 4°C until assay. The supernatants were assayed for LDH and analysed for reduced Alamar Blue.

### 2.7.8 End point evaluation of HK-2 cells maintained under perfusion and static culture conditions

HK-2 cells were grown to confluence on fibronectin-coated polycarbonate filters in filter carriers. At time 0, filter carriers were transferred either to static (1 filter carrier per well of a six well plate with 3 ml medium) or perfusion conditions for 24 or 72 h. Medium used for both conditions was defined DMEM Ham's F12 with 5 mM glucose. At the end of 24 or 72 h, Alamar Blue was assayed on each filter as described. LDH, GGT and protein were assayed in cell homogenates. All perfusion and static experiments were carried out in a paired fashion, in 6 independent experiments, with 3 to 6 replicates. Data was analysed for statistical difference using a two-tailed paired t-test.

## 3 Results

### 3.1 Perfusion culture equipment and handling

*Flow rate:* The delivery rate of the Ismatec peristaltic pump was tested using deionised water over a number of 24 h periods. Three different tube lines and chambers were assembled and tested on each of the 8 pump positions. The weight of the water delivered over three 24 h periods was compared. There were no significant differences in delivery rates between the 8 pumping positions of the Ismatec pump (Fig. 3). A maximum of 3% variation was observed when using repeatedly autoclaved pump tubing (not shown). Thus, pump tubing should be re-

placed when wear is noticed. All pump tubing should be replaced at the same time as excessive intra-experimental flow variances may affect toxicological data extrapolation.

### 3.2 Temperature monitoring

It is suggested by the suppliers of the perfusion system that the cell culture perfusion chamber be placed on a heating plate to keep the cultures at 37°C throughout experimentation. However, many laboratories do not have constant temperature control and therefore the heating plate may not be adequate to maintain a constant internal chamber temperature. To investigate the temperature of the internal perfusion chamber under perfusion conditions, an electronic temperature sensor probe was inserted into the top of one chamber. Temperature fluctuations were monitored over a period of 5 days with the chamber placed on the heating plate or in an enclosed 37°C incubator (Stuart Scientific). Deionised water was continuously perfused through the chamber at 1 ml/h. The reservoir for the deionised water was maintained at ambient room temperature. As can be seen from Figure 4, temperature fluctuation was greatly reduced when the perfusion chamber was maintained in an enclosed 37°C incubator. The use of an

enclosed incubator may be more important for laboratories which have high room temperature fluctuations. This experiment was conducted during the autumn months under non-constant room temperature control.

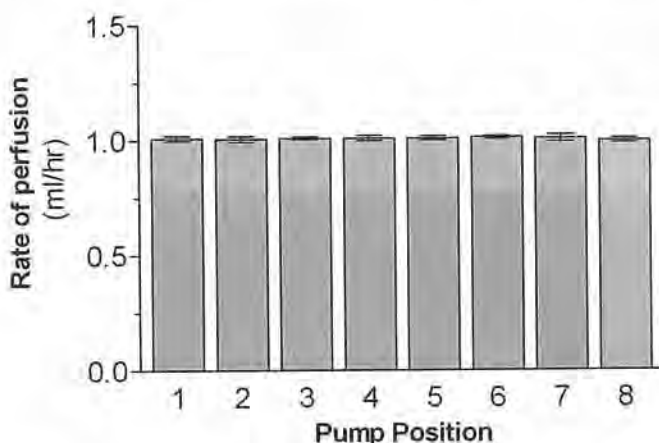
### 3.3 pH control

According to the Henderson-Hasselbach equation, the pKa of the cell culture medium is dependent on the partial pressure of CO<sub>2</sub> and the quantity of sodium bicarbonate. The tubing lines of the perfusion system provided are silicone and thus are permeable to air. Medium equilibrates with air as it passes from the reservoir bottle to the perfusion chamber. Normal cell culture medium containing 14 to 22 mM bicarbonate will be alkaline by the time it reaches the chamber. We were reluctant to increase the HEPES content in the medium above 20 mM in order to maintain osmolarity within physiological levels. It was demonstrated that medium containing 20 mM HEPES and 5 mM sodium bicarbonate maintained pH values close to pH 7.4 ( $\pm 0.1$ ) under ambient CO<sub>2</sub> conditions. However, in order to carry out appropriate paired controls, both static (conventional non-perfused cell cultures maintained in a 5% CO<sub>2</sub>, 37°C humidified environment) and perfusion cell culture medium should be

the same. Thus, silicone tubing was replaced with CO<sub>2</sub>-tight tubing (Pharmed, ColePalmer) and reservoir medium bottles were bubbled slowly with 5% CO<sub>2</sub> balanced with air. DMEM-F12 containing 14 mM bicarbonate and 15 mM HEPES was used for both perfusion and static culture experiments. In this way the extra-cellular pH was maintained between pH 7.3 and pH 7.5.

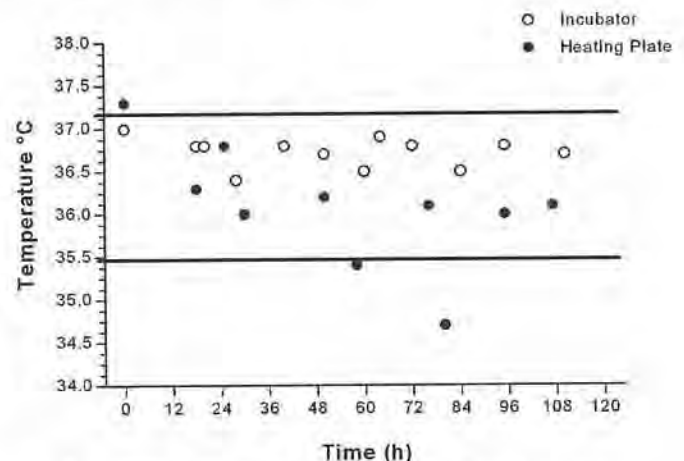
### 3.4 Perfusion chamber design modifications

Initial studies using the original chambers demonstrated a high variance in cell growth and vitality parameters between the different perfusion positions. It was thought that this variance might be due to a lack of homogeneity of medium flow in the chamber. In an attempt to improve this situation the chamber was modified in order to allow individual medium inlets between the filter carriers. This added a complication to the system in that small air bubbles entering the system would readily block the passage of medium in subsequent inlets. Thus a bubble trap was designed and introduced (Fig. 2 B, C). The bubble trap allowed the removal of air bubbles at the main inlet and thus the subsequent inter-filter carrier inlets remained free of trapped air.



**Fig. 3: Variation in fluid delivery between different pump positions.**

De-ionised water was delivered through the perfusion system at each pump position for a period of 24 h with the same tubing up. The weight of the water delivered was measured and mean rate of perfusion was calculated. Results represent the mean of 3 experiments  $\pm$  SEM. No difference was observed between positions.



**Fig. 4: Temperature variation comparison in the perfusion chamber over time with the hot plate or in an enclosed incubator.**

Deionised water was perfused at 1 ml/h through the adapted chamber with a temperature probe. Temperature was recorded over five days with the chamber on a heating plate or in an enclosed incubator. Lines represent physiological temperature borders.



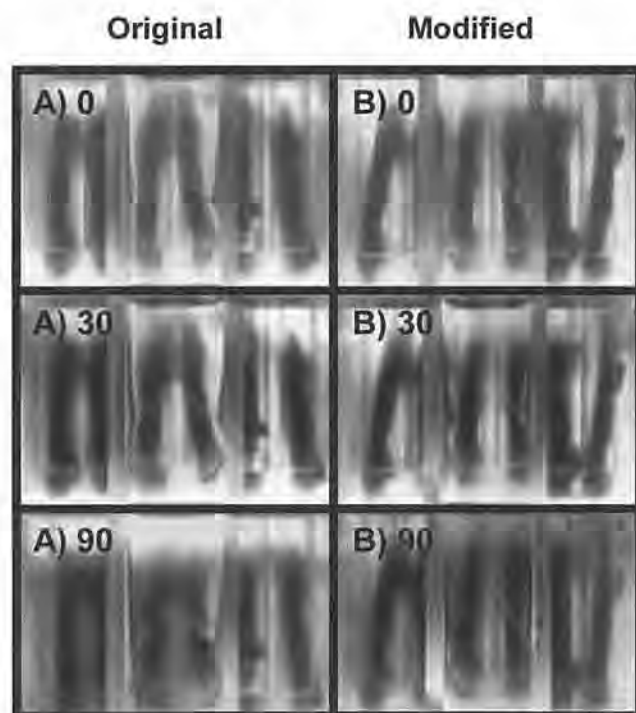
### 3.5 Homogeneity of medium distribution

Modification of the perfusion chamber to allow medium to enter the chamber at seven points on the base of the chamber between the filters (as opposed to the original single entry point at the left of the chamber) altered the dynamics of flow distribution (Fig. 5). In the original chamber, the coloured medium fills the chamber from left to right. Thus the first filter is always exposed to fresh medium, whilst the last filter is exposed to medium, which has already passed 6 filters. In the modified design, the coloured medium enters at the base of the chamber, filling the cham-

ber from bottom to top between the filter carriers. Thus each filter receives approximately the same amount of new medium. Also, it can be seen at 90 min that the coloured dye mixes better with the colourless dye in the modified chamber than in the original chamber (Fig. 5). It is concluded from this experiment that the chamber modification introduced improved flow distribution within the chambers. It is worth noting that the chamber volume is 5 ml with all filter carriers inserted. Thus it will take at least 5 h (at 1 ml/h) before the concentration of chamber dye (analogous to toxin) reaches the concentration of the incoming dye.

### 3.6 Biochemical comparison between filter carrier positions

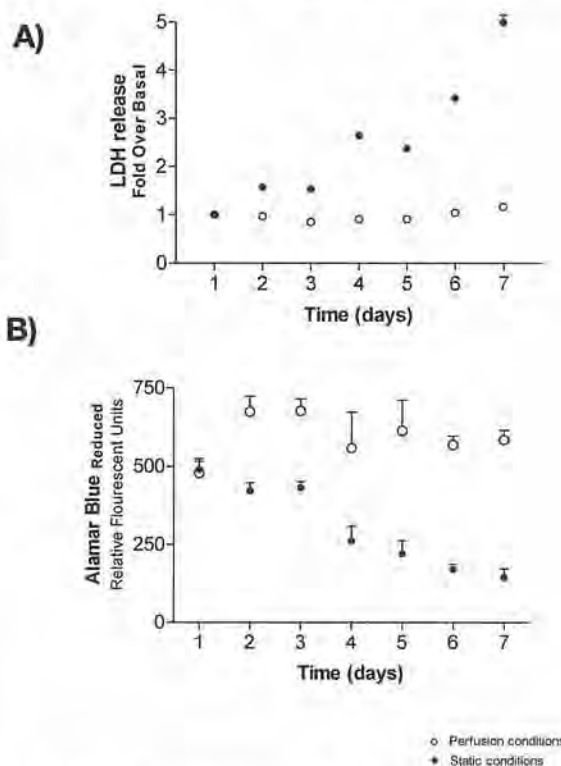
HK-2 cells were maintained under perfusion conditions for 72 h in modified or unmodified chambers. Viability was assessed for each filter using the Alamar Blue assay. Cell homogenates were prepared and assayed for LDH activity. LDH activity demonstrated a position-related activity in the original chamber (Fig. 6A). As the filter carrier position moved further away from the inlet, the activity of LDH was lower. Statistical analysis gave a linear correlation of  $r^2 = 0.90$ , with a slope of  $-0.08$ , which was significantly non-zero ( $P=0.0034$ ).



This figure can be found in colour at the website [www.altex.ch](http://www.altex.ch)

**Fig. 5: Photographs of perfusion chambers containing filter carriers, at 30 min and 90 min after an exchange of colourless medium with blue medium, in the original (A) and modified chamber (B).**

Cell culture medium (DMEM-Ham's F12) without phenol red was exchanged at time 0 with identical medium with 0.08 g/L methylene blue added, at a rate of 1 ml/h at 37°C. The filter carriers contained polycarbonate clear membranes without cells. Photographs were taken every 10 min using back light illumination of the chambers. The photos shown here were taken from the front of the semi-transparent chamber and are representative of 3 independent experiments.



**Fig. 7: Comparison between LDH release and Alamar Blue reduction under perfusion and static conditions over 7 days in culture.**

HK-2 cells grown on collagen-coated aluminium oxide filters were maintained for 7 days under perfusion conditions (6 filter carriers per modified perfusion chamber, flow rate 1 ml/h, bubbled with 5% CO<sub>2</sub>, 95% air) or static conditions (6 filter carriers per 10 cm Petri dish with 20 ml medium, unrefilled throughout experiment, in humidified 5% CO<sub>2</sub> incubator). Cell culture medium contained Alamar Blue at a concentration of 0.2% v/v. 100 µl samples were drawn from static culture every 24 h. Medium was also collected from out-flow of the perfusion chamber for 1 h every 24 h. A) Lactate dehydrogenase release from perfusion and static culture as a function of time. B) Alamar Blue reduction from perfusion and static culture as a function of time. Values represent the means of two experiments + SEM.

There was no statistical correlation between position and LDH activity for cells maintained in the modified chamber. There was no correlation between filter carrier position and Alamar Blue reduction for either group (Fig. 6B). However, the coefficient of variance was higher in the original chamber than in the modified chamber (13.07% and 7.24% respectively). Also, the mean Alamar Blue reduction (all filters pooled) was significantly higher in the modified chamber as compared to the original chamber,  $801.4 \pm 33$  fluorescent units and  $624.9 \pm 33$  fluorescent units respectively with  $P=0.0015$ . Since it was demonstrated using dye exchange that the distribution of the medium under perfusion conditions is improved in the modified chamber, it was not surprising to find a more heterogeneous cell activity in the unmodified chamber as compared to the modified chamber. However it was a surprising result that the LDH activity was higher in the unmodified chamber, depending on how close the filter was to the inlet. Since LDH and Alamar Blue were to be used as parameters for toxicity, it was important that the variance in these parameters between each filter in the chamber remained minimal. Thus all further experiments were carried out using the modified chamber design.

### 3.7 Comparison between static and perfusion culture conditions

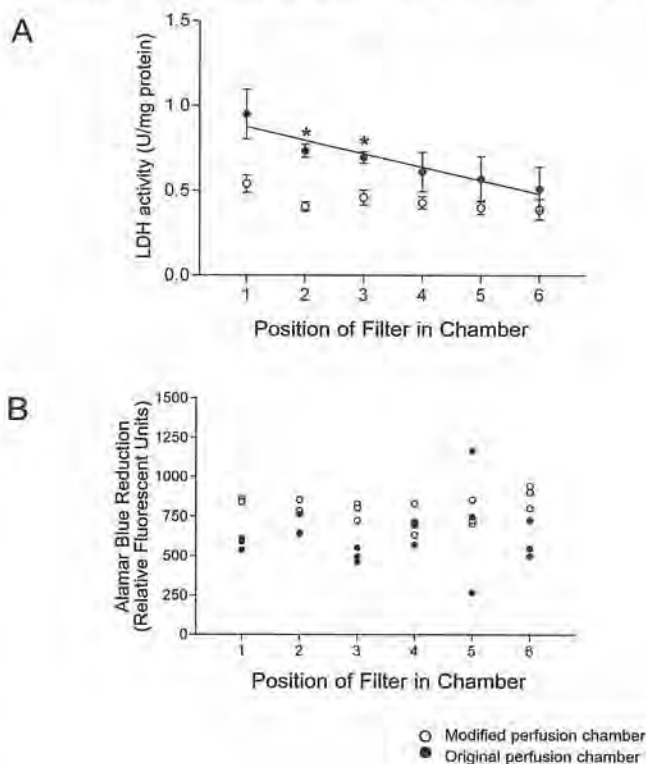
Basal release of LDH into the medium and Alamar Blue reduction were compared under static and perfusion conditions (Fig. 7). The medium of the static cell cultures accumulated LDH linearly as a function of time. Under perfusion conditions, although there was a detectable amount of LDH activity present, there was a continuous amount of supernatant LDH activity over time. LDH is released normally in small amounts by the cells under both conditions, but accumulates in static culture and is continuously removed under perfusion conditions. The supernatant LDH activity measurement illustrates that cultures maintained under continuous perfusion are subjected to a more stable environment than under static conditions.

Alamar Blue is a REDOX indicator, which is converted from a non-fluores-

cent blue compound to a fluorescent red compound by viable cells. Both non-reduced and reduced Alamar Blue freely permeates cell membranes and is reported to be non-toxic. Thus the addition of non-reduced Alamar Blue to cell culture medium and the subsequent detection of reduced Alamar Blue in the cell supernatant is a measure of cell vitality (Fields and Lancaster, 1993; Nociari et al., 1998). This assay was of interest in the present study due to reports that it is a safe, easy and simple end point viability assay (Ahmed et al., 1994) and due to the possibility of using this assay in real time cell viability monitoring under perfusion conditions. The production of reduced Alamar Blue remained relatively stable over time under perfusion conditions, but declined linearly as a function of time

under static conditions (Fig. 7B). It was surprising that Alamar Blue reduction should decrease under static conditions, as once all Alamar Blue is reduced to the fluorescent form, fluorescent measurements would have been expected to stay constant over time despite cell viability.

End point Alamar Blue reduction, protein content, LDH activity and GGT activity were compared in cells maintained under static or perfusion conditions for 24 and 72 h (Fig. 8). There were no differences between perfusion and static conditions in any of the parameters measured at 24 h (not shown). Alamar Blue reduction and LDH activity were significantly lower under perfusion conditions at 72 h than under static conditions. Protein and GGT activity was the same under both conditions at 72 h.



**Fig. 6: Biochemical comparison between chamber filter position of HK-2 cells maintained in perfusion conditions in original or modified chambers.**

HK-2 cells on fibronectin-coated polycarbonate filters were maintained under perfusion conditions for 72 h in either original perfusion chambers or modified perfusion chambers. Perfusion conditions were: hormonally defined medium bubbled with 5% CO<sub>2</sub> balanced with air, perfused with 1 ml/h at 37°C. LDH activity of cell homogenates and Alamar Blue conversion was determined for each filter position. (A) LDH activity is expressed as mean values of units/mg protein  $\pm$  SEM. \* denotes statistical significant difference from modified chamber group using a 0.05 threshold. (Exact P values were 0.058, 0.002, 0.013, 0.206, 0.277 and 0.447 for chamber positions 1 to 6 respectively). Continuous line is a linear regression correlation with  $r^2=0.91$ . (B) Alamar Blue reduction is expressed as individual values in arbitrary fluorescent units.



## 4 Discussion

The main focus of this study was the assessment of the applicability of perfusion culture systems to toxicity studies. Some important limitations of the commercially available perfusion system were recognised. In order to allow a scientifically sound evaluation of the toxicological application of this perfusion culture system, it was necessary to modify the system itself. The modifications and adaptations as outlined below allowed cells cultured under perfusion conditions to be maintained under stringent conditions of temperature, pH and homogeneity of flow. This was necessary in order to allow direct comparisons with the static model and to avoid influences of factors beyond experimental control to interfere with the interpretation of experiments.

### 4.1 Temperature

Unreasonable fluctuations of cell culture temperatures for even short periods of time may alter toxicological sensitivity of cells by the induction of heat shock proteins (HSP). HSP 70 is induced in LLC-PK<sub>1</sub> cells by 2 h of incubation at 42°C, conferring tolerance to cyclosporine A (CsA) exposure for up to 24 h after shock (Yuan et al., 1996). In other studies, over-expression of HSP 72 induced tolerance to both cisplatin and oxidative injury (Komatsuda et al., 1999). Hypothermia has also been shown to induce the expression of HSP 72 in mice (Cullen and Sarge, 1997). The perfusion system allows control of temperature by placing the perfusion chambers on a heating plate. The heating plate in our laboratory had to be set to 42°C in order to maintain internal perfusion chamber temperature at approximately 37°C. This method of temperature con-

trol was deemed unacceptable due to high fluctuations in temperature under our laboratory conditions. Maintaining the perfusion chambers in an enclosed 37°C incubator improved temperature stability considerably (Fig. 4).

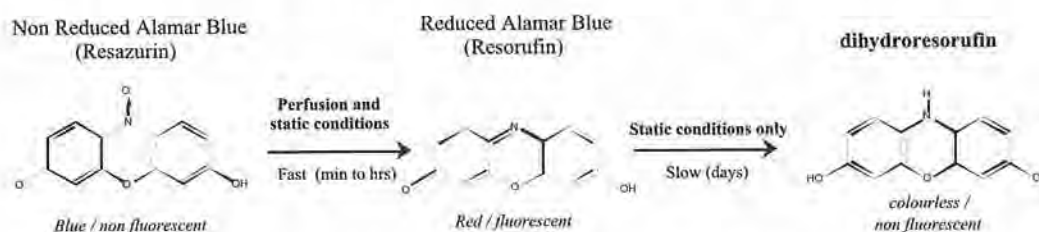
### 4.2 pH control

In order to carry out non-perfusion controls as well as possible it was necessary to change the pH buffering method of the perfusion system to one compatible with a normal cell culture incubator. Thus the perfusion apparatus was adopted from ambient CO<sub>2</sub> buffering to 5% CO<sub>2</sub> buffering by bubbling the reservoir bottles with 5% CO<sub>2</sub>, 95% air and changing the tubing lines from CO<sub>2</sub>/air permeable silicone to CO<sub>2</sub>/air impermeable Pharmed. Thus the same medium (with normal bicarbonate concentrations) was used for both perfusion and static experiments. This method also ensures that the medium entering the perfusion chambers is oxygen-saturated.

### 4.3 Flow distribution

In order to carry out efficient toxicity evaluation with the perfusion culture system it was necessary that each of the six filter carriers in the perfusion chamber could be considered a replicate. For this to be true there should be no position-related advantages or disadvantages to the cell monolayers. However, this was not the case. Perfusion of medium from one inlet at the lower corner of the chamber with an exit at the upper corner on the opposite side of the chamber did not supply medium homogeneously across all 6 of the cell monolayers (Fig. 6). Thus the perfusion chamber was redesigned to allow medium to enter the base of the chamber between each filter carrier (Fig. 2C). In order to fully

investigate the modification in chamber design a simple experiment was conducted in which HK-2 cells were grown on filters in filter carriers and transferred to 3 modified perfusion chambers and 3 original chambers. Each chamber was maintained under identical perfusion conditions for 72 h. In the original chambers LDH activity per mg protein demonstrated a position relationship, with monolayers closer to the inlet having higher specific activity (Fig. 6A). LDH activity in LLC-PK<sub>1</sub> has been shown previously to be influenced by glucose concentration and oxygen delivery (Gstraunthaler and Handler, 1987; Gstraunthaler et al., 1999). Thus a nutritional and/or oxygen gradient induced in the original chamber may have been responsible for the position-related response. One possibility is that the utilisation of glucose by the cells closer to the inlet creates a slight glucose gradient, thus cells closer to the outlet have less glucose available. There was no correlation between position and specific LDH activity using the modified chambers. This is strong evidence of a better homogeneity of medium in the modified design. There was no correlation with endpoint Alamar Blue reduction and filter position for either perfusion chamber type (Fig. 6B), however the coefficient of variance was higher in the original chamber as compared to the modified chamber, 13.07% and 7.24% respectively. Thus the variability of end-point Alamar Blue reduction is also much improved using the modified perfusion chamber. In conclusion, the modification of the perfusion chamber design leads to more homogenous conditions within the chamber, thus allowing each filter carrier within a chamber to be considered a replicate for endpoint determinations.



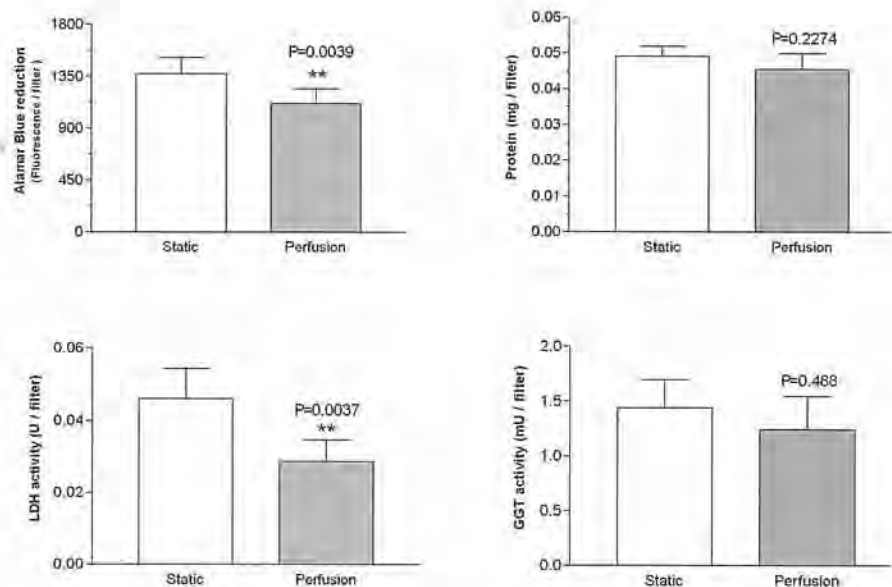
**Fig 9: Diagrammatic representation of the conversion of Alamar Blue under static and perfusion conditions.** (See text for details).



#### 4.4 Dynamic difference between perfusion and static cultures

In the non-perfused cultures there was a time-dependent accumulation of supernatant LDH (Fig. 7A). LDH is widely utilised as a marker of cell membrane integrity. Thus, basal LDH release may be considered a marker of natural cell turnover (In cell culture without the presence of macrophages, apoptotic cells will become secondarily necrotic). Basal cell death was not detectable under perfusion conditions. This has important consequences for chronic toxicity testing, since a low level of toxicity may be amplified under non-perfusion conditions due to the release and accumulation of normally intracellular enzymes from damaged cells. Under perfusion conditions (as in the body) such amplification would only be immediately local.

The production of reduced Alamar Blue by HK-2 cells was shown to be reasonably stable over time under perfusion conditions. However it was puzzling that there was a decrease in Alamar Blue fluorescence over time under static conditions (conversion of Alamar Blue to the reduced form is irreversible and reduced Alamar Blue is stable). The identity of the dye substance in the Alamar Blue solution has not been disclosed by the manufacturers. However the properties of the assay have a striking resemblance to a resazurin-based assay, which is also commercially available (Sigma R-6892). Recently it was demonstrated that the active ingredient in Alamar Blue solution is indeed resazurin (O'Brien et al., 2000). These authors also demonstrated in primary rat hepatocytes that resazurin (non-reduced Alamar Blue) which is reduced to resorufin (reduced Alamar Blue) by viable cells is finally reduced to dihydroresorufin, a colourless non-fluorescent compound. This phenomenon would explain the time-dependent loss of fluorescent signal from static cultures. This also presents an interesting example of dynamic differences between the interaction of cells in perfusion and static culture with exogenous compounds, which are metabolised. Since the reduction of resazurin to resorufin is fast (min to h) there is little difference between initial reduction rates under perfusion and static



**Fig. 8: Comparison of biochemical parameters of HK-2 cells grown under perfusion or static conditions for 72 h.**

HK-2 cells were grown to confluence on fibronectin-coated polycarbonate filters in Minusheet carriers. At day 0 carriers were transferred to perfusion chambers (6 carriers per chamber) or to 6 well plates (one carrier per well). 6 well plates were incubated for 72 h in 3 ml defined medium per well in a humidified 5% CO<sub>2</sub>, 37°C incubator. Carriers in perfusion chambers were perfused for 72 h with defined medium at a delivery rate of 1 ml/h. All experiments were paired for time and date, fibronectin coating, cell population, and medium components. At the end of 72 h the cells were assayed for Alamar Blue conversion and lysed. Lactate dehydrogenase activity (LDH), protein, and gamma glutamyl transpeptidase activity (GGT) was determined in cell lysates. Results represent the mean + SEM of 6 independent experiments each performed with 3 to 6 replicates. All values are quoted per filter. Statistical analysis was performed using a 2-tailed paired t-test. Paring was deemed effective for all data using a Pearson correlation coefficient test.

ic conditions (Fig. 7 B). However this process quickly becomes saturated under static conditions when all the Alamar Blue has been converted. The reduction from resorufin to dihydroresorufin is relatively slow (days), thus this process only occurs under static conditions (Fig. 9).

#### 4.5 Biochemical comparison between perfusion and static cultures

The advantage of continuously supplying cell cultures with fresh medium is not completely clear. On the one hand, there is a removal of waste products and a constant supply of nutrients such as glucose (illustrated by the removal of basal released LDH from HK-2 cells). On the

other hand, there is a removal of potentially important paracrine factors and hormones. In this study we observed two significant differences between 72 h perfusion-maintained, filter-grown HK-2 cells and 72 h static-maintained filter-grown HK-2 cells. At the end of the 72 h incubation, cells under perfusion conditions had decreased cellular LDH activity and converted Alamar Blue less efficiently than statically maintained cells, whereas GGT and protein levels were the same for both groups (Fig. 8).

A reduction of activity of the glycolytic enzyme LDH has been reported for proximal tubular cells before. In general, conditions of increased aeration decrease LDH activity. LLC-PK<sub>1</sub> cells grown in



roller bottles, which promote better oxygenation of the cells, have reduced LDH activity compared to static conditions (Sahai et al., 1989). Addition of heptanoate to cultured rabbit proximal tubules to support oxidative metabolism decreases glycolytic enzyme activity (Aleo and Schnellmann, 1992). Also increasing media volumes in Petri dish-grown LLC-PK<sub>1</sub> cells, which decreases oxygen diffusion, increased LDH activity (Gstraunthaler et al., 1999). Thus, reduced LDH activity of HK-2 cells under perfusion conditions is probably related to a better oxygen supply. This is a reasonable assumption considering that medium bubbled continually with 5% CO<sub>2</sub>, 95% air is continuously perfused into the perfusion chamber.

Since there is less Alamar Blue conversion under perfusion conditions, it might be believed that the cells are less viable when maintained under perfusion conditions than under static conditions. O'Brien et al. (2000) have demonstrated in HepG2 cells that Alamar Blue conversion occurs in the cytosol and not in mitochondria. We have demonstrated here that there is a down-regulation of the glycolysis-associated enzyme LDH under perfusion conditions. Reduced glycolytic activity of proximal tubule cells has been associated previously with an increase in mitochondrial metabolic enzymes such as phosphate-dependent glutaminase (Cole et al., 1986). Thus it is possible that under perfusion conditions there is an altered cytosolic REDOX state, since the cells may have an increased oxidative metabolism.

In summary, the perfusion system was optimised successfully for the maintenance of renal cell cultures. Temperature, pH and flow rate could be regulated at acceptable levels. The homogeneity of flow was improved by a modification of the system, which allowed all of the filters within one chamber to be considered as replicates for end-point measurements. LDH activity in cell culture supernatant accumulated and reduced Alamar Blue (resorufin) was converted to a non-fluorescent form (dihydroresorufin) under static conditions but not under perfusion conditions. Thus, there is a subtle dynamic difference between static and perfusion conditions.

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