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## **A method for continuous and stable perfusion of tissue and single cell preparations with accurate concentrations of volatile anaesthetics**

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**Running head:** Method for continuous delivery of volatile anaesthetics to tissue samples

### **Keywords**

Volatile anaesthetics, perfusion rig, infrared analyses, gas chromatography-mass spectrometry

## Abstract

*Background* It is difficult to design a system to reliably deliver volatile anaesthetics such as halothane or isoflurane to in vitro preparations such as tissues or cells cultures: the very volatility of the drugs means that they can rapidly dissipate from even carefully-prepared solutions. Furthermore, many experiments require the control of other gases (such as oxygen or carbon dioxide) which requires constant perfusion.

*New Method* We describe a constant perfusion system that is air-tight (ie, allows the accurate administration of hypoxic or hypercapnic gas mixtures), in which volatile anaesthetic is delivered via calibrated vaporizers by constant bubbling into the perfusing solution (and continuously monitored for stability by infrared spectroscopy in the headspace above the solution).

*Results* We have confirmed the accuracy (ie, linear relationship of dissolved concentrations with vapour dial settings) and stability (ie, over time) of the anaesthetic concentrations in solutions in samples taken from the bottles into which anaesthetic is bubbled, and from samples taken from the tissue perfusion bath, using gas chromatography-mass spectrometry (GC-MS).

*Conclusions* It is possible to deliver volatile anaesthetics in accurate concentrations to cell/tissue preparations whilst adjusting ambient air composition rapidly, stable over sustained time periods.

## Highlights

- Due to the volatility of some anaesthetic gases, it has been proved difficult to design a system quantitatively to deliver these agents in stable concentrations to in vitro preparations.
- We describe a reliable system for the accurate administration of volatile anaesthetics to cell and tissue samples, with minimal loss of vapour and consistency of anaesthetic stimulus.
- In contrast to previously described ‘closed’ systems, our ‘open’ system uses continuous bubbling of anaesthetics in the perfusing solution and continuous monitoring of the headspace gas of above the solution with infrared analyses. GC-MS measurements demonstrate consistent concentrations in our experimental tissue perfusion bath.

## Introduction

In a reductive approach to understanding the mechanism of action of volatile anaesthetics, it is often necessary to perfuse tissue cultures or single cell preparations with solutions equilibrated with these agents and study their responses, such as changes in intracellular calcium or membrane electrophysiology. Sometimes, it is further necessary simultaneously to change the background gas ( $O_2$  and  $CO_2$ ) tensions to study the interactions of anaesthetic with hypoxia/hypercapnia (Pandit and Buckler, 2009, Pandit et al., 2010) as relevant to many questions in clinical practice (van den Elsen et al., 1994, Pandit, 2002, 2005).

Handling volatile agents in standard delivery systems (perfusion ‘rigs’) poses several challenges. Their very volatility (boiling points  $\sim 23$ - $59^\circ C$ ) means that these gases have a tendency to be lost from solution. Even if it is air-tight, the delivery system needs to ensure sustained equilibrium between anaesthetic gases in solution with the gas phase above the solution. This is because the biological effect of anaesthetics is determined by their partial pressure rather than absolute content in solution (Eger et al., 1986). However air-tight the delivery system, experimental baths into which the tissue or cells are placed are impossible to shield completely from the atmosphere and rapid evaporation can occur if this is not well controlled. Furthermore, some tissue/cell preparations need to be constantly perfused with  $O_2$  or nutrients to maintain constancy of their environment, or there is often a need to adjust the prevailing  $O_2$  and/or  $CO_2$  gas tensions (eg, switches between euoxia and hypoxia and/or hyperapnia are necessary). These aims are not possible to achieve simply by immersing the preparation in a fixed volume of pre-prepared liquid.

To date, it has been proved difficult to design a system that meets all these requirements in delivering anaesthetics to *in vitro* preparations. In a typical set up, the anaesthetics are delivered to a physiological solution (eg, Tyrode) with vaporizers to achieve equilibration. For such a set-up, (Franks and Lieb, 1993) and (Smith et al., 1981) described a method for estimating the concentration in solution (in mM) of anaesthetic delivered from a calibrated vaporizer (where the delivery of agent is marked in percent, corresponding to a partial pressure), using the relevant Bunsen or Ostwald water/gas partition coefficient for the agent in question. Following the equilibration by bubbling, the solution is then stored in bottles or syringes ‘air tight’ from between a couple of minutes to several hours, to be delivered later to an experimental bath (which is inevitably exposed to the atmosphere). How this equilibrated agent is stored and delivered to the biological preparation differs across

published studies. One convenient way of storing the pre-equilibrated solutions is in Erlenmeyer flasks covered tightly with parafilm (Sirois et al., 1998). Generally, these methods might be described as ‘closed’, since their aim is to keep the anaesthetic in solution closed to the atmosphere until the time of exposure to experimental tissue.

Despite being ‘closed’, chromatographic measurements of the samples from the experimental baths using these methods have revealed a loss of 20% - 50% compared with what was expected from calculations based on the dialled concentrations on the vaporizers (Miu and Puil, 1989, McDougall et al., 2008). Furthermore, because the experimental bath is inevitably exposed to the atmosphere, (McDougall et al., 2008) demonstrated that a very variable loss of agent can arise unless there is a system for continuous perfusion with equilibrated solution. The option of frequent sampling from the experimental bath solution on a daily basis to confirm system performance is not only very time consuming, but also expensive.

In contrast to such ‘closed’ methods of delivery, (Conway and Cotten, 2012) described an ‘open’ system. They directly applied gas vaporised with anaesthetics to the solution perfusing the experimental bath, and monitored the gas phase of the anaesthetics in the head space above the bath by continuous infrared analyses. A similar set-up was also described by (Jinks et al., 2005). However, these systems did not allow for control of the gas composition ( $O_2$  and  $CO_2$ ) perfusing the tissue.

In this paper we describe an extension to Conway et al.’s and Jinks et al.’s systems, which can both be described as ‘open’ in the sense there is continuous administration of anaesthetic to solution rather than prior storage of agent. Yet, our system is also ‘closed’ in the sense of being air-tight, as one additional aim is to maintain conditions that enable us to administer near-anoxic stimuli to the cells of interest, so that it is suitable for work with cells like carotid body glomus cells that are oxygen-sensing. Here we describe the new system and its performance in achieving these aims.

## Methods

### *Perfusion system*

The cell perfusion system is shown in Figure 1. Gas is delivered from compressed gas cylinders connected through air flow regulators and rotameters via narrow-bore plastic tubing to vaporizers containing anaesthetic. The gas cylinders (British Oxygen Company, Surrey, UK) can contain air,  $N_2$ ,  $O_2$ , and  $CO_2$  in various concentrations of hypoxic mixtures (we

commonly use  $N_2$  in 5%  $CO_2$  or 1%  $O_2$  with 5%  $CO_2$ ), or hypercapnic mixtures of air with various concentrations of  $CO_2$  (20% being common). The typical flow rate of gas delivery to the vaporizers is 200-500ml/min, measured via calibrated rotameters. From the vaporizer outlet the anaesthetic-gas mixture passes through narrow-bore flexible nylon tubing (BS5409) tubing to a plastic costar pipet gas dispersion tube (Corning, New York, USA) immersed in a glass bottle (volumes of 0.2-2 L are used, as needed) containing the experimental salt solution (Tyrode, in mM: 117 NaCl, 4.5 KCl, 1  $MgCl_2$ , 23  $NaHCO_3$ , 11 glucose). The bottles are capped with a plastic lid (Schott Duran, Wertheim, Main, Germany), into which are punched two holes: one large hole to allow passage of the gas dispersion tube, and one small hole to allow passage of the narrow bore metal aspiration tube (see below) that aspirates the solution into the delivery system for perfusion to the experimental bath. This small hole also allows passage of a fine plastic connector tube leading to the infrared gas analyser (Capnomac Ultima, Helsinki, Finland) for continuous anaesthetic vapour analysis of the headspace above the solution in the bottle. This analyser was checked and calibrated with readings from a mass spectrometer (Airspec 3000, Airspec Ltd, Biggin Hill, UK; not used continuously during this experiment), in turn calibrated with standard agents as calibration gases.

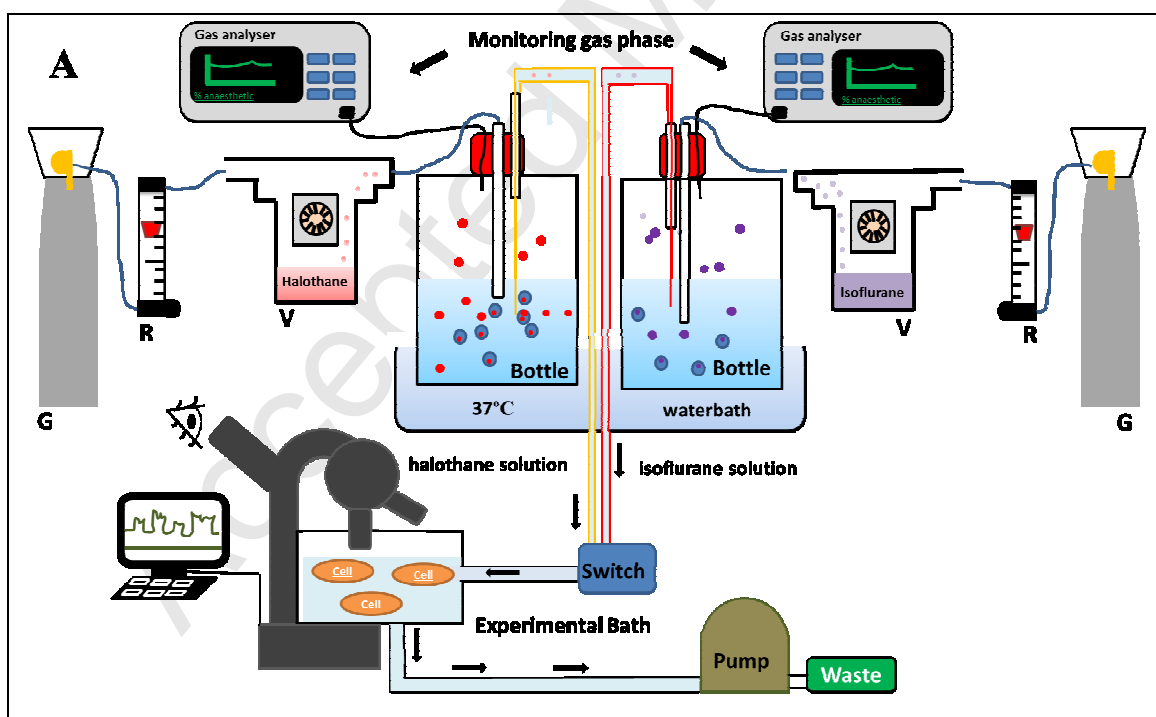
The bottles are placed in a water bath heated to  $\sim 38^\circ C$  to achieve a temperature of  $37^\circ C$  in the experimental bath. The gas from the vaporizer bubbles continuously into the bottle throughout the experimental period, with sufficient time for equilibration before the start of any experimental recordings, as confirmed by attainment of the desired, stable value on infrared capnography. Both (Millman and Young, 1992) and (Becker et al., 2012) have shown that equilibration is reliably achieved after  $\sim 10$  min across a range of anaesthetic concentrations.

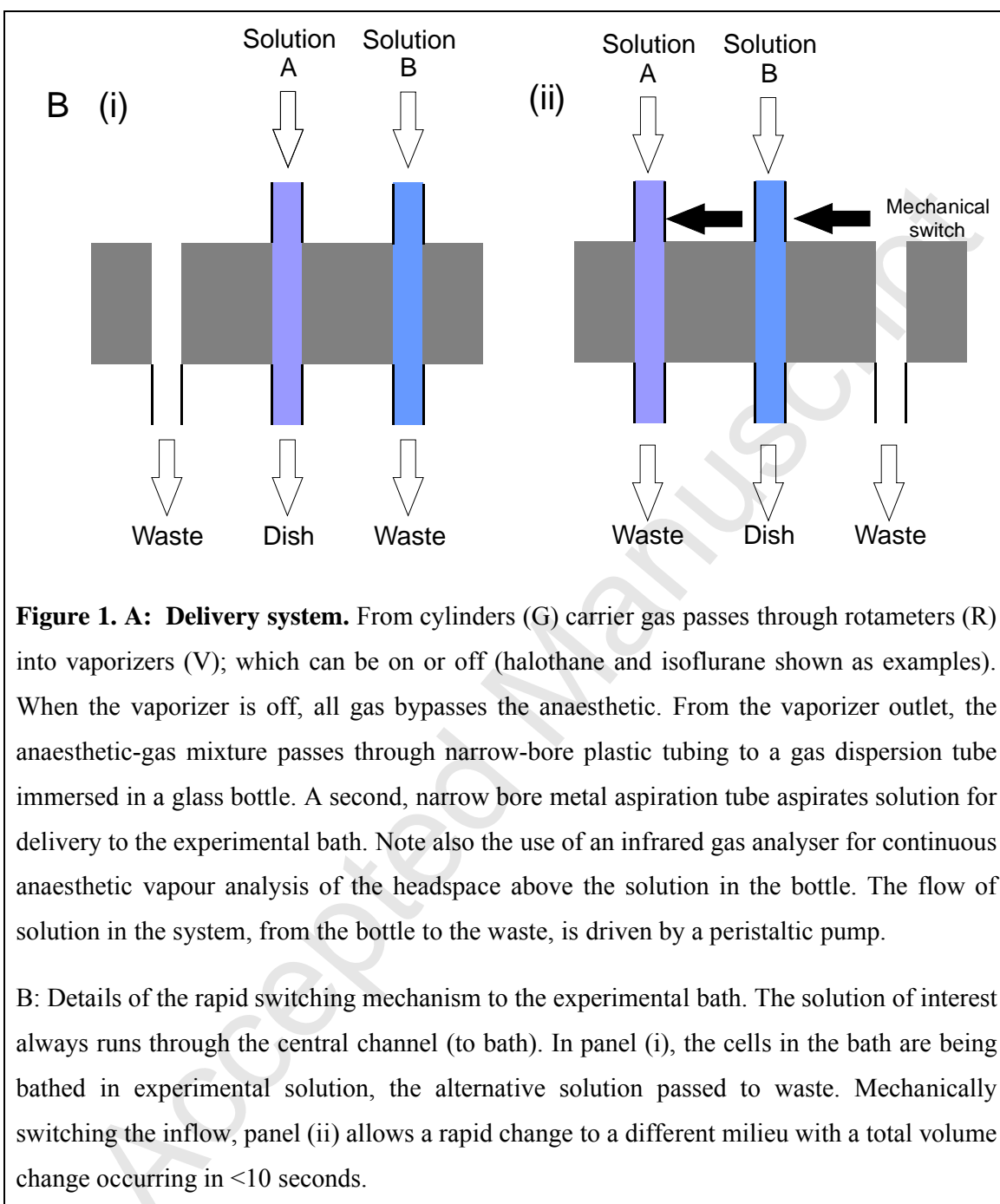
To deliver the solution from the bottle to the experimental bath, a stainless steel (medical grade) aspiration tube is placed into the solution of the glass bottle via a small hole in the cap (see above). The delivery to the experimental bath is by gravity through stainless steel tubing and short sections of Pharmed (Akron, USA) tubing (flow 6-7ml/min). The solution is then aspirated from the bath using a peristaltic pump as a vacuum source. It is possible to make a switch from one experimental solution (eg, air) to another (eg, air with anaesthetic) using a two-way tap (constructed of PTFE and nylon) connected to the bath entry, which is operated by a lever (Figure 1B). Switching the tap results in rapid change of solution within  $<1$  s. The solution exits the experimental bath and passes through compressors of a peristaltic pump. The experimental bath is itself moulded from Perspex in-house, in its centre being a depression (volume 100  $\mu l$ ) in which sits the coverslip containing

the cells or tissue sample. Its base is clear glass, allowing focussing of the cells using an inverted microscope.

#### *Assessment of delivery of anoxic gas mixture*

We first assessed the air-tightness of our system by measuring  $O_2$  levels in the experimental bath. The rationale was that if air tight, then little or no  $O_2$  would enter the perfusion system if an anoxic (ie, pure  $N_2$ ) mixture was delivered. The  $O_2$  levels were measured with an optical  $O_2$  sensor (Presence, Regensburg, Germany) equilibrated with a normoxic and anoxic gas resulting in a two-point calibration. The normoxic gas used was 5%  $CO_2$  in 95% air and the anoxic equilibration gas used was 5%  $CO_2$  in 95%  $N_2$ , with the addition of 250 $\mu$ M  $Na_2S_2O_2$  (sodium dithionide), which reacts with and so consumes all the remaining  $O_2$  in the solution, so that an anoxic solution is ensured for calibration. We then used two test gas mixtures: severe 'hypoxia' 0% (5%  $CO_2$  in 95%  $N_2$ ) and 'mild' hypoxia 1% (1%  $O_2$ , 5%  $CO_2$ , 94%  $N_2$ ).





#### *Sampling for chromatography measurements of anaesthetic in solution*

Samples were taken from two sites in the apparatus (Figure 1). The solution from (a) the bottle into which anaesthetic was bubbled and (b) from the experimental bath was carefully but rapidly drawn up using a 2 ml syringes and then swiftly placed into 2 ml autosampler vials (Finneran Associates, Vineland, USA) until a convex meniscus was formed. The cap was attached immediately excluding any air contamination. The vials were



then analysed in batches. We separately confirmed using repeated measurements of the same sample over 24 hours that there was no loss of anaesthetic from the autosampler vials, verifying that measurement in batches was appropriate.

#### *GC-MS measurements*

GC-MS analysis was performed using an Agilent 7200 Quadrupole Time-of-Flight mass spectrometer, coupled directly to an Agilent 7890B Gas chromatograph and an Agilent GC Sampler 120 autosampler (Agilent, Stockport, UK). Standards were prepared in a non-polar solvent so as to exploit the high oil:gas partition coefficients of halothane and isoflurane (224 and 98 respectively). Toluene (analytical grade, Fisher, Loughborough, UK) was chosen as it has a higher boiling point (111 °C) than either halothane or isoflurane (50 and 48 °C respectively) and would elute after the analytes when using standard gas chromatography techniques.

The autosampler was equipped with a 10 µL syringe and its operation was carefully designed to minimise outgassing of analytes. Prior to injection, the syringe was flushed once with toluene then three times with water. The syringe was then cleaned with the sample twice (10µL of sample was withdrawn and dispensed to waste). The syringe filling speed was set to 0.5µL/s and sample was drawn up and ejected three times before injecting 10µL of the sample. Following injection, the syringe was flushed three times with toluene and three times with water.

The gas chromatograph was equipped with a HP-5 column (30m, 0.25mm ID, 0.25µm film thickness, Agilent, Stockport, UK), the carrier gas was helium (BOC, Slough, UK) and the flow rate was 1.2ml/min. The inlet was set to 300°C with a split ratio of 100:1. The oven was held at 40°C for three minutes then ramped at 100°C/min to 240°C and held for 2 minutes. The transfer line was maintained at 300°C.

The mass spectrometer was operated in electron ionisation (EI) mode with a source temperature 230°C and electron energy of 70eV. Mass spectra were collected between 50-500 m/z and ionisation was disabled after 3.7 minutes (after all analytes had eluted but before the toluene eluted).

MassHunter software (Agilent, Stockport, UK) was used for instrument control and data analysis. Responses for each analyte were determined using the area of peaks seen in the total ion count (TIC) mass chromatograms. Identities of analyte peaks were confirmed

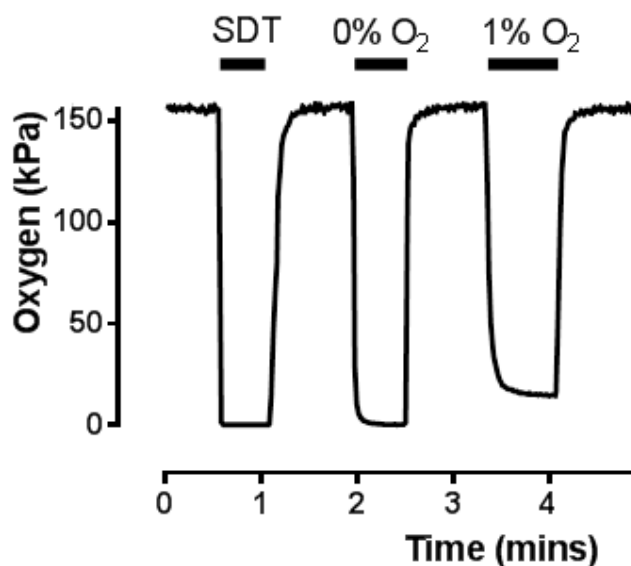
using comparison to the NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA)

## Results

### *Assessment of delivery of anoxic gas mixture*

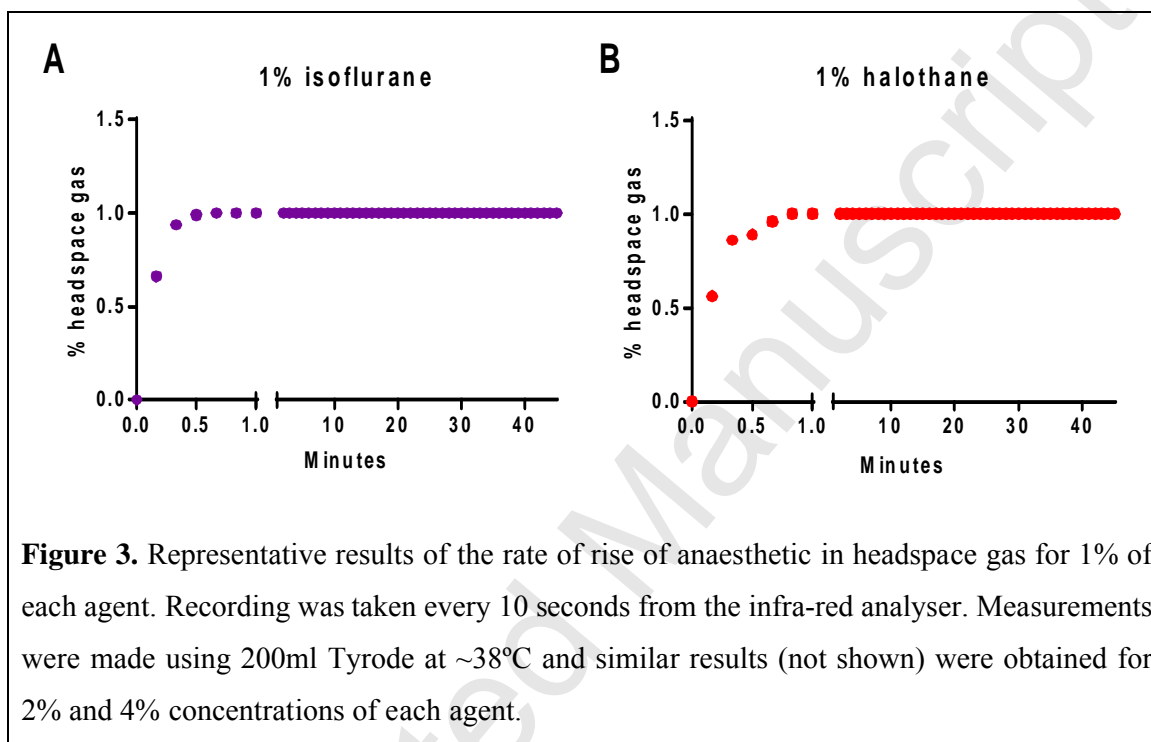
When a mild hypoxic gas mixture (1% O<sub>2</sub> bubbled; predicted pO<sub>2</sub> in experimental bath 7.6 mmHg) was delivered, the electrode placed in the experimental bath measured 10.6 mmHg. When severe hypoxia (5% CO<sub>2</sub> in 95% N<sub>2</sub>) was used, the electrode measured 3 mmHg. These results confirmed that the apparatus was air-tight with negligible ingress of O<sub>2</sub> from the atmosphere. Furthermore, since we sampled directly from the experimental bath, this also indicated that our flow rates were appropriate and maintained the hypoxia. Furthermore, switches into and out of anoxia/hypoxia were rapid, with the new equilibrium achieved within 3 sec (Figure 2).

Figure 2. Delivery of anoxic gas mixture. Estimates of pO<sub>2</sub> in solution in the experimental bath against delivered O<sub>2</sub> concentration. The first gas input (SDT) is severe hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) with sodium dithionite, which elicited 0 mmHg. Note the rapid fall and equilibration of hypoxic gas mixtures.



*Anaesthetic sampling*

Figure 3 shows that the rate of rise of anaesthetic for each agent in the headspace was rapid and reached equilibrium after just a minute, and remained stable thereafter for >40 minutes that were tested. Few experimental periods last longer than this.



Whereas anaesthetic concentrations were stable for as long as vapour was bubbled and the system was in equilibrium, sometimes protocols require long interruptions to anaesthetic exposure followed by re-exposure. The solution could be bubbled continuously with vapour when not in use, but this wasteful of anaesthetic. Therefore we also assessed the time course for washout of agent from solution (headspace) in two protocols after equilibration as described above. First, we turned the vapourisers off but allowed agent-free gas to bubble into solution. Figure 4A shows that vapour loss from headspace was very rapid with a half-time of ~50 seconds regardless of agent or initial concentration. Second, we turned off both vapour and bubbling; we did not seal the bottle air-tight but allowed natural leakage from the small holes in its stopper. We measured headspace gas at 15 minute intervals and Figure 4B shows that under these conditions washout was very slow (Figure 4B), suggesting that re-equilibration in re-introducing anaesthetic would be faster with this method.

Figure 4. Washout of agent when anaesthetic bubbling stopped. A: Time course of loss of headspace vapour when anaesthetic turned off but agent-free bubbling continued. Initial concentrations of 2% and 1% are shown for halothane (red) and isoflurane (purple). For clarity, fitted lines only shown, rather than individual data points. B: Time course of loss of headspace vapour when both anaesthetic and bubbling turned off (starting concentrations 1% for both agents). Note the much longer timescale on x-axis.

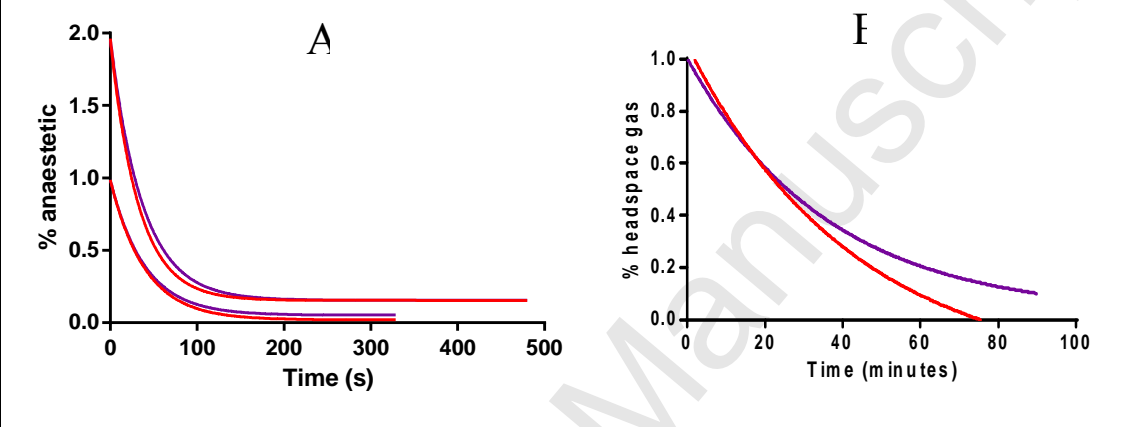


Figure 5 shows the results of sampling for the two anaesthetic agents from the bottle, and Figure 6 shows the results for sampling from the experimental bath. It is clear that for both agents there is a strong linear relationship between the vaporisation rate on the vaporizer and the concentration (parts-per-million, ppm) obtained in solution, across a very wide range of concentrations ( $r^2$  range 0.93-0.98). These relationships were found for both sampling from the bottle and the experimental bath. Exact equations were calculated (see Figure legends), but approximately the relationship for our apparatus in the experimental bath is ~5 ppm per % vapour, regardless of agent. The variation in results was highest at the higher concentrations, perhaps indicating the influence of sampling error at these higher doses.

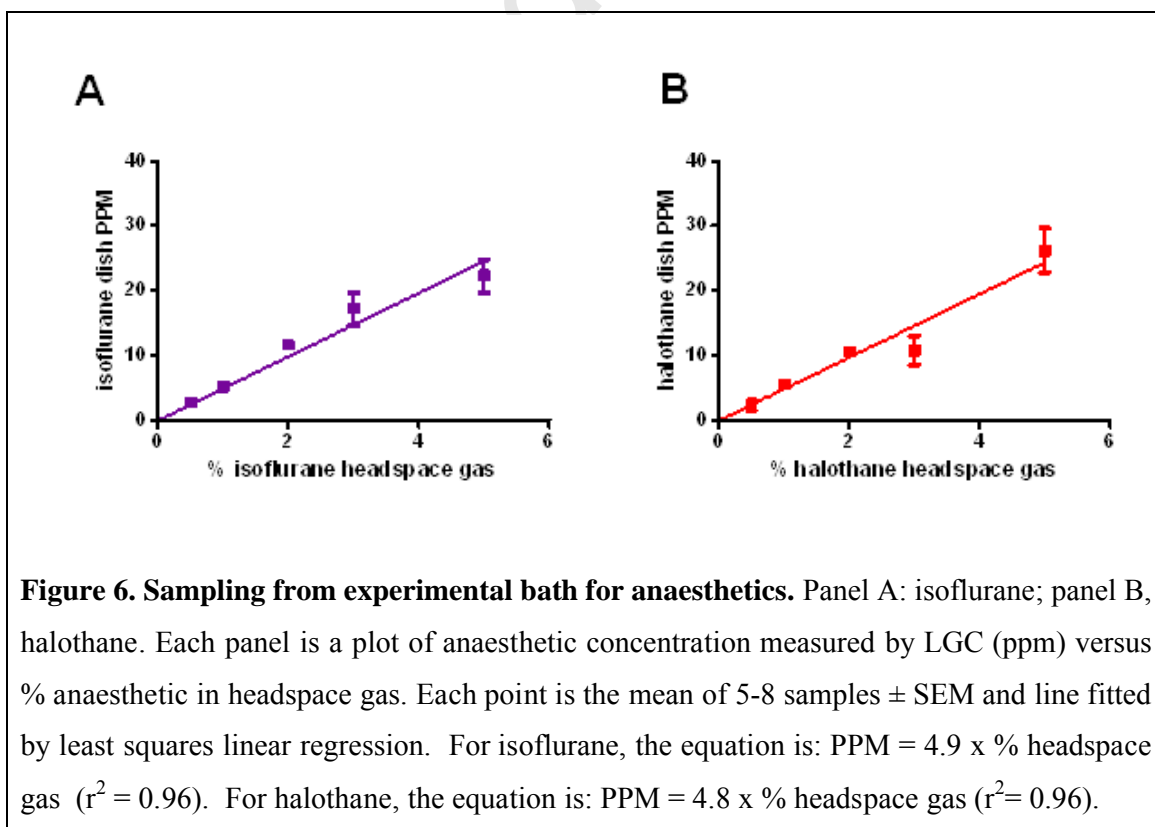
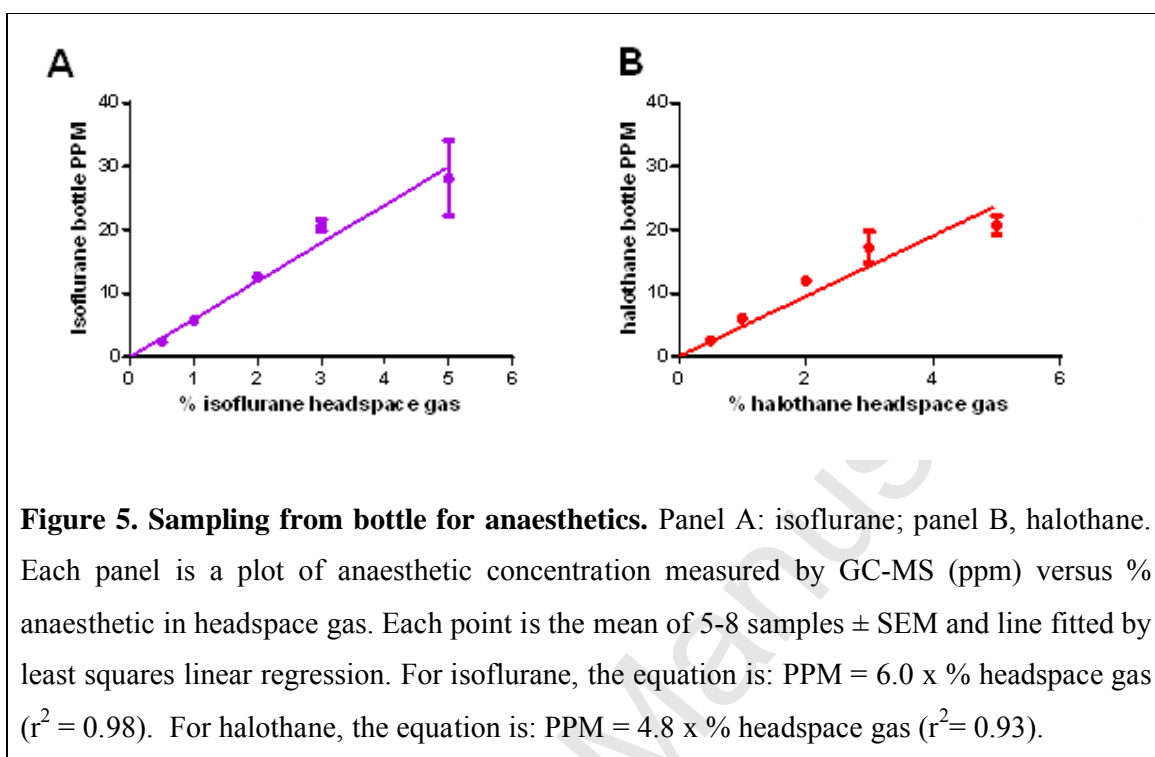
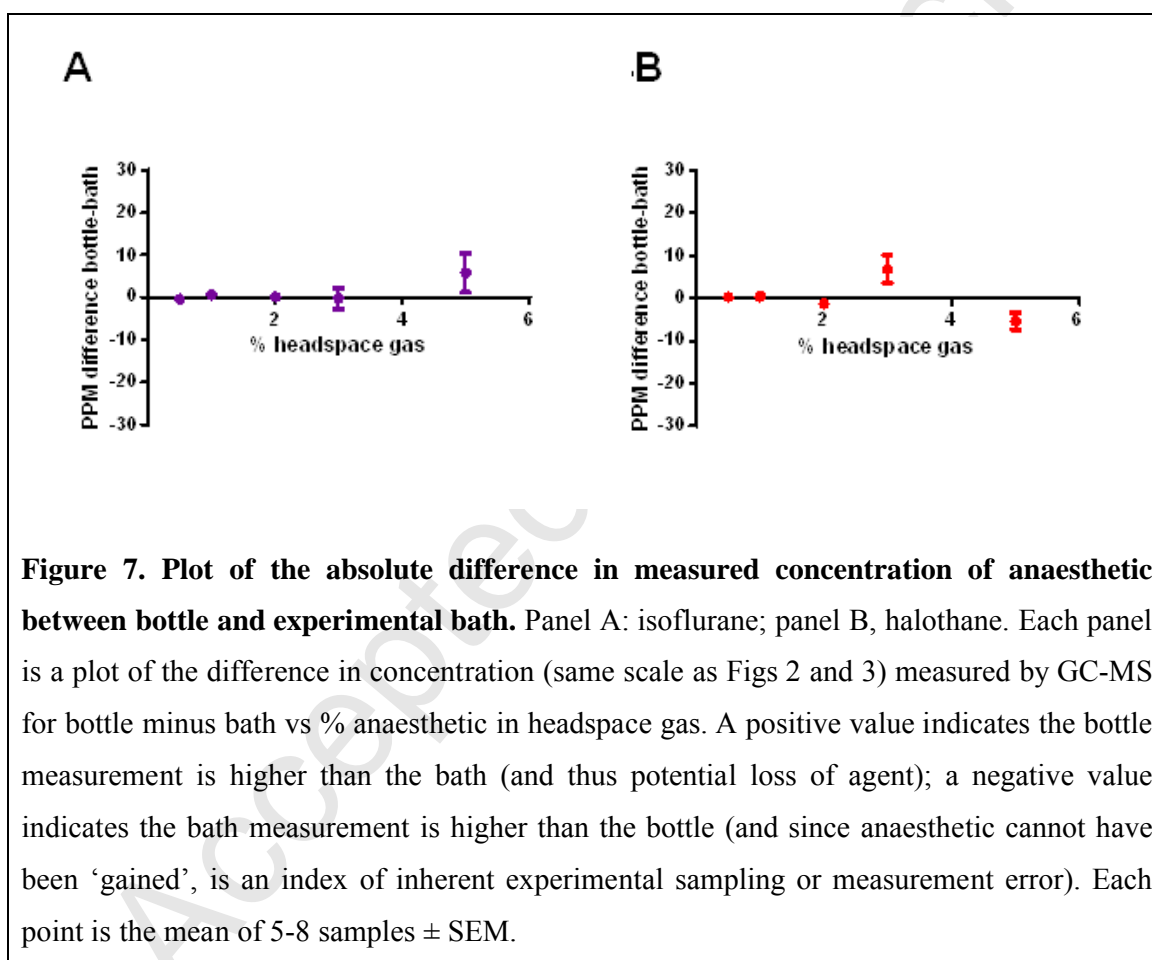


Figure 7 shows that there was no consistent loss of agent across a wide range of concentrations between bottle and perfusion bath for both agents. Statistically this was assessed using factorial analysis of variance, where the difference in concentration of agent between bottle and bath was the ‘response’ and there were two factors: ‘agent’ (two levels, one for each anaesthetic) and ‘dose’ (five levels, one for each concentration). None of the iterations of ‘dose’, ‘agent’ or the interactive term were significant ( $p > 0.127$ ), confirming no consistent difference between bottle and bath concentrations either across concentrations or between agents.

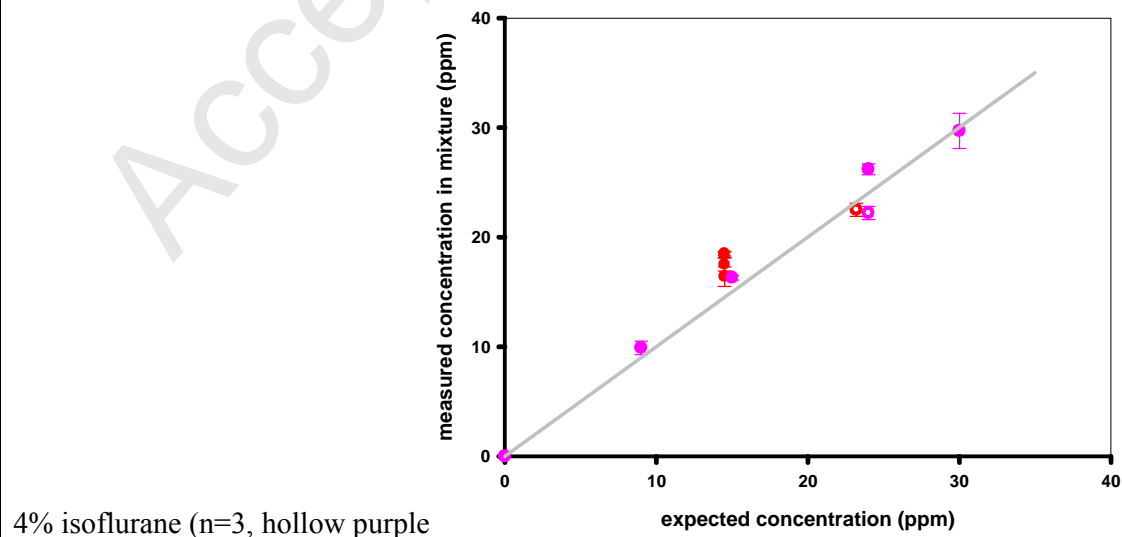


We conducted additional experiments in which we bubbled mixtures of agent into solution by placing the vaporisers in series. Anaesthetic mixtures are sometimes used to confirm the additive interactions of agents on cell functions (Hendrickx et al., 2008) but infra-red analysers are unable accurately to measure a mix of agents. We assessed the behaviour of the system in three ways. First, employing three-way taps in the gas lines connecting the vaporisers, we used infra-red analysis to measure continuously the output of

each vaporiser separately. This confirmed that dialled concentrations of the proximal vaporiser were not influenced by the presence of a second, distal vaporiser turned on and in series with the first (ie, no unanticipated back-pressure effects). We used infra-red analysis to confirm that the output of the second, distal vaporiser was not influenced by turning on an empty, proximal vaporiser (ie, no forward-pressure effects). Second, we used the mass spectrometer (which can separately detect and quantify different agents in a mixture) to confirm that concentrations in the headspace in the mixture were the same as dialled on the vaporisers when mixtures of agent were used, across a range of concentrations. Third, we performed GC-MS measurements as described above, taking samples from the bottle. Figure 8 confirms that agents dissolve in solution in mixtures, as expected from their individual behaviour.

Finally we tested for the presence of thymol in halothane samples but none was detected in any of the samples using GC-MS (with an accuracy of <1parts per billion; ppb).

Figure 8. Concentration of mixtures of anaesthetics in solution. Data are shown (mean  $\pm$  SEM). The expected concentrations are calculated from the relationships in Figure 65 (line of identity shown). The solid circles represent data for a fixed concentration of halothane (2.5%) combined in separate experiments with varying concentrations of isoflurane (1.5%, n=5; 2.5%, n=5; 4%, n=5; and 5%, n=3). Thus the purple solid circles represent the data plotted for isoflurane and the red solid circles data for halothane in the mix (overlapping points for halothane). Also shown is an example of a mixture of 4% halothane (n=3, hollow red) and



## Discussion

We have described that our system for the ‘open’ and continuous perfusion with volatile anaesthetic of cells/tissue in our experimental bath achieves stable and reliable concentrations of agents in solution. We have confirmed this for halothane and isoflurane, but there is no reason to suppose that other agents would be differently affected. Importantly, agent concentrations in solution, in the perfusion bath where the cells are bathed, are linearly related to vaporizer dialled concentrations, and with very little variation across a wide range of concentrations (Figures 5 and 6).

One limitation is that our method only yields anaesthetic concentrations in the clinically relevant range, the limits set by the range of the vaporizers. It also requires that the outputs of the vaporizers are calibrated for gas flow and/or that outputs are continuously measured (eg, by infrared gas analysis) since vaporizer outputs are sensitive to flow through them. An additional limit is set by the published accuracy range of the infra-red analyser (which for the type we used is 0-5% for each agent). If anaesthetic concentrations outside the vaporizer/analyser range need to be studied, then there is little alternative other than to premix agents in a ‘closed’ system. However, we are not aware of such techniques being used in combination with the effects of changes in gas composition.

The variation in measured concentrations in solution from ideal (Figure 7) were more likely be due to our methods of sampling than any shortcomings within the apparatus, because for some concentrations, the bath concentrations were actually slightly higher than in the bottle. It is difficult to sample from this small bath (volume 100  $\mu$ l). Or, it could represent some inevitable diffusion of vapour from the system. These minor variations were not so large as significantly to influence the linear relationship between desired and actual concentrations (Figures 5 and 6). It is this linear relationship that is all-important when constructing concentration-response curves to demonstrate drug effects. Indeed, sampling error is a more plausible explanation for this variation as our system is clearly extremely air-tight, because near-anoxic conditions were maintained in the perfusion dish when O<sub>2</sub>-free gas (‘severe’ hypoxia) was used. Adsorptive loss to Teflon or metal tubing has been previously demonstrated not to occur (Herchl, 1970, Suzuki et al., 2005).

We continuously also monitored the concentrations of anaesthetic vapour from the headspace gas above the solution in the bottle, and our verification of the accuracy by GC-MS confirms that therefore, this should be a generally sufficient method of monitoring the input vapour concentrations. Repeated GC-MS measurements are laborious, expensive and clearly unnecessary for our system (although intermittent verification is probably



recommended, especially if the apparatus components are refined or renewed). In many experiments involving anaesthetics, it is more important to describe the overall concentration-effect relationships for the cell preparation in question, than it is to ascertain a given effect at an exact anaesthetic concentration. Only if the latter is essential will it be necessary to perform GC-MS on a very frequent basis.

Although we generally perform studies with continuous bubbling of vapour, other experiments might necessitate intermittent interruptions to perfusion by agent (eg, anaesthetic exposure pre- or post-conditioning to assess hypoxia/reperfusion injury). Our data for washout suggest that anaesthetic concentration will be better retained – for less than ~30 min - if bubbling is stopped during this period, facilitating more rapid re-equilibration (Figure 4A). If bubbling is continued without vapour, even for less than a minute, loss of agent is very rapid (Figure 4B).

In terms of the absolute value (in ppm or  $\mu\text{M}$ ) of anaesthetic concentration in solution for a given percentage vaporizer concentration, a very wide range indeed has been reported in the literature. We measured (in ppm) ~50 – 150  $\mu\text{M}$  in solution for ~1-3% anaesthetic in the vapour phase (for both agents), which is a slightly wider range, starting at a lower concentration, than that reported by (McDougall et al., 2008) of ~100-180  $\mu\text{M}$  for similar vapour concentrations, and close to the ranges reported by (Miu and Puil, 1989), who noted that measured concentrations were in fact less than half the value of those predicted by calculated partition coefficients. However, all these values are approximately half as much again as those reported by (Becker et al., 2012), and a factor of nine times less than found by (Roch et al., 2006) and by (Pancrazio, 1996). We cannot explain these large disparities, except to suggest that they may all be related to the precise composition of solutions used in the respective studies and their solubility for anaesthetics, or sampling error, or vapour loss, or a combination of all of these. In our study, we would expect the intrinsic vapour loss to be greatest between the bottle and the experimental dish, and we would also expect sampling error to be greatest for sampling from the dish, yet these losses were trivial (Figure 7). Therefore, we are minded to conclude that the data represent the true solubility of the specific Tyrode solution composition we employed.

We also note that the biological action of volatile anaesthetics is regarded as driven by their partial pressure, and not their dissolved content (Eger et al., 1986). Thus by way of a ‘bioassay’ we have previously reported the potent inhibition (through an effect on TASK channels) of the intracellular calcium response in carotid body glomus cells to hypoxia at halothane vapour concentrations of ~1.5%, becoming maximal at ~3% (Pandit and Buckler,

2009), which we estimate to be ~150-300  $\mu\text{M}$  in our solution. Similarly, Sirois et al. (2000) reported near-maximal effects TASK-channel  $\text{K}^+$  currents at identical partial pressures, although they had employed a 'closed' system of prior dissolution of halothane in air-tight flasks, at higher absolute dissolved concentrations of ~500  $\mu\text{M}$  (Sirois et al., 2000). Our open system therefore resembles how volatile anaesthetics are administered in clinical practice to tissues of the body: the bottle into which agent is first bubbled represents the 'lungs' (Figure 1), and the remainder of the system (the solution, the tubing, the pump) represents the blood, cardiovascular system and heart respectively, delivering agent to tissues. Thus, the biological effects upon tissues we examine would not theoretically be influenced by replacing our Tyrode solution with, say, blood even though the absolute anaesthetic carrying capacity of the latter would be much larger.

Finally, we note the complete absence of thymol in solution when halothane is vaporised into it. Thymol is added to halothane at a concentration of 0.01% as a stabiliser and preservative. When the vaporizers are not drained as advised every week, thymol can accumulate (Rosenberg and Alila, 1984). It has been suggested that thymol may be an active ingredient in some of the properties of halothane since it was found to influence GABA-A receptor activity in mouse cortical neurons (Garcia et al., 2006) and decreases the L-type  $\text{Ca}^{2+}$  and potassium current in canine cardiomyocytes (Magyar et al., 2002). We can now robustly conclude that these results are purely a function of applying halothane mixtures directly to cell preparations in vitro. When halothane is vaporised using conventional equipment, there is simply no vaporisation of thymol. We believe therefore, that this is one additional advantage of our experimental system, in that it avoids the potential confounder that, if premixed solutions of halothane are used, the thymol effects that others have reported may be seen.

In summary, we report a reliable system for the accurate administration of volatile anaesthetics to cell and tissue samples, with minimal loss of vapour and consistency of anaesthetic stimulus. Furthermore, our system is air tight such that extremely low levels of hypoxia can be co-administered. Finally, rapid switches into and out of hypoxia/euoxia and/or from one agent to another or to no agent can be achieved.

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