

Application note

Controlled normoxic, hypoxic, and low-oxygen incubation using VitroFlow.Bio

Generation of controlled gas environments during continuous microfluidic perfusion using the Be-Flow Standard chip by Beonchip

Executive Summary

Precise gas conditioning is essential for microfluidic cell culture, where small channel volumes, high surface-to-volume ratios, and continuous perfusion make dissolved gas stability particularly sensitive to temperature, pressure, medium composition, and chip handling. This application note evaluates the gas-conditioning performance of the VitroFlow.Bio CO₂ incubation module during continuous perfusion using the Be-Flow Standard chip by Beonchip. The system was assessed by downstream dissolved oxygen monitoring during 72 h incubation at 5% CO₂, comparative measurements at 5%, 10%, and 20% CO₂ against a conventional incubator, lower-limit testing at 100% CO₂, flow and sterility monitoring, and preliminary biological testing with *Rhynchomonas* sp. protozoa under reduced-oxygen conditions.

At 5% CO₂, dissolved oxygen reached a stable plateau of approximately 7.300 µg/L after 6 h and remained stable throughout the 72 h experiment. Across 5%, 10%, and 20% CO₂, VitroFlow.Bio produced oxygen concentrations comparable to a standard CO₂ incubator, while showing lower spatial variance. Under 100% CO₂, dissolved oxygen approached zero after approximately 22.1 h, with a maximum O₂ depletion rate of 35.74 µg/min. Peristaltic tubing maintained stable flow generation for up to 16 days, and protozoa remained visually viable under 100% N₂ and perfusion at 100 µL/min. These findings show that VitroFlow.Bio provides controlled gas conditioning for normoxic, hypoxic, and near-anoxic microfluidic incubation while preserving perfusion, temperature control, and live microscopy access for future low-oxygen organ-on-a-chip and environmental-sample applications.

Keywords: VitroFlow.Bio, microfluidics, organ-on-a-chip, perfusion cell culture, gas conditioning, CO₂ incubation, dissolved oxygen, hypoxia, microfluidic incubation, live-cell imaging

1. Introduction

Precise environmental control is essential for mammalian cell culture, particularly in microfluidic systems. Besides temperature regulation, defined CO₂ conditions are required to maintain bicarbonate-buffered media and physiological pH stability. In organ-on-a-chip systems, this control must be achieved within small-volume channels while maintaining continuous medium supply, dissolved gas conditioning, and experimental accessibility. A conventional solution is to place the full setup (pumps, tubing, sensors, actuators, and chip) inside a CO₂ incubator. Although well established,

this approach limits microscopy, sensor access, manual handling, and long-term hardware reliability, as warm and humid incubator conditions can promote corrosion and technical failure. Microfluidic incubation is particularly sensitive to bubble formation because low volumes and high surface-to-volume ratios amplify gas exchange, temperature gradients, and pressure-related solubility shifts. Even small bubbles can interrupt perfusion, alter shear stress, or block the culture region. If the dissolved gas composition of the medium differs from the equilibrium gas saturation state of the chip or tubing, equilibration can locally exceed solubility limits and lead to bubble formation.

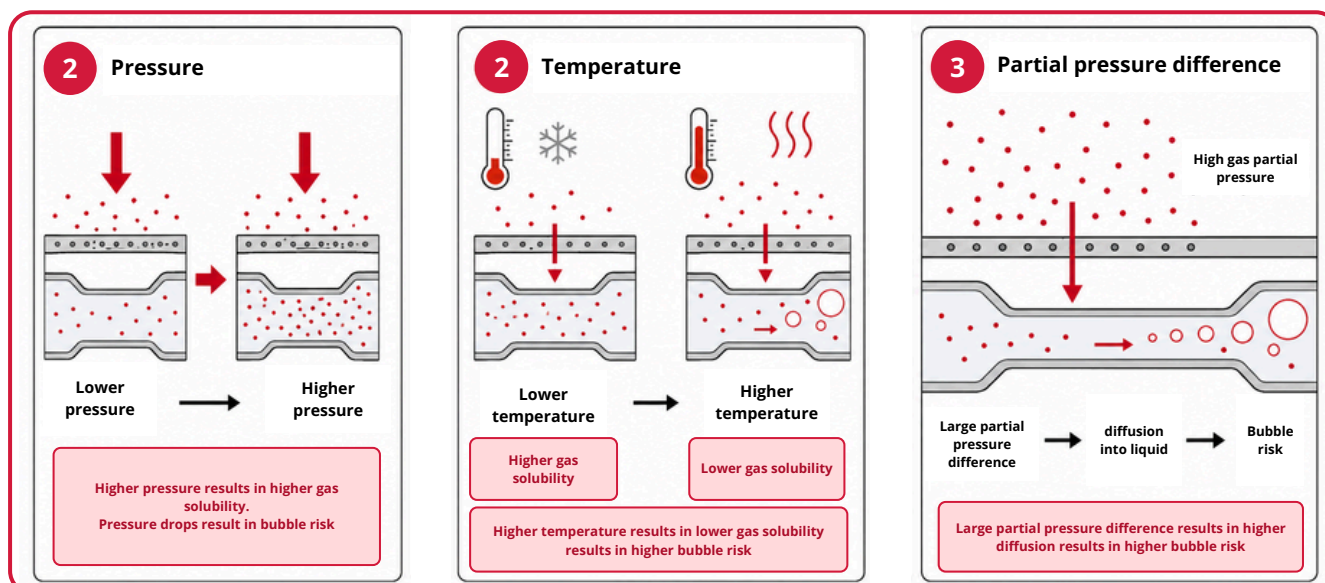


Figure 1: Factors affecting gas solubility in water and water-based media, illustrating **1.** the effect of solubility of gas increasing with the system's pressure; **2.** the effect of decreasing solubility with increasing temperature; and **3.** the effect that strong differences in partial pressure between phases can induce bubble formation.

Accordingly, the Be-Flow guide recommends overnight equilibration of the chip, tubing, and fluidic elements before seeding or flow setup. Temperature and pressure further affect dissolved gas equilibria. Gas solubility in liquids is temperature-dependent, so medium conditioned at one temperature may release gas when exposed to warmer regions in the chip or tubing (Battino and Clever, 1966; Sander, 2023).

$$c_i = k_H \times p_i$$

Ambient pressure changes also shift gas solubility, as exploited in vacuum degassing, and can become relevant in sensitive low-volume systems (Battino and Clever, 1966). Reliable microfluidic incubation therefore requires not only a defined gas atmosphere, but also a system architecture that minimizes uncontrolled equilibration between gas conditioning and the chip. To address this, VitrofluidiX developed a microfluidic CO₂ incubator as part of the VitroFlow system. VitroFlow.Bio combines controlled perfusion, a regulated CO₂ atmosphere, and temperature control with four individually controlled flow lanes, an external microscope-compatible chip chamber, integrated bubble traps, flow sensors, and a monitored heated CO₂ incubation module. The VitroFlow incubator provides the possibility to establish controlled gas atmospheres containing up to 100% CO₂, with gas monitoring at ±0.5% accuracy and an inner volume of 650 µL ± 50 µL. Unlike full-setup incubation, VitroFlow separates medium gas

conditioning from chip handling. The medium is conditioned inside the VitroFlow system before being perfused to the chip, while the chip remains temperature-controlled, pressure-free, accessible for microscopy, and protected by upstream bubble traps. Material-related gas equilibration remains chip-dependent, so preparation should be performed according to the manufacturer's recommendations.

This application note evaluates the gas-conditioning performance of the integrated VitroFlow.Bio CO₂ incubation module using the Be-Flow Standard chip from Beonchip. The Be-Flow contains two independent perfusable channels with screw-like inlet/outlet wells. The Be-Flow Standard device is made from gas-impermeable materials, enabling precise control of oxygen levels within the microchannels and making them particularly suitable for hypoxia and controlled-oxygen models. The study demonstrates 72 h operation at 5% CO₂, explores system limits at 100% CO₂ against a conventional incubator reference, and complements sensor-based validation with *Rhynchomonas* sp. protozoa cultured in-chip under a CO₂/N₂ atmosphere. As *Rhynchomonas* has been reported among anaerobic flagellates (Priya et al., 2008), it provides a relevant model for low-oxygen microfluidic cultivation. The aim is to assess whether VitroFlow.Bio provides controlled microfluidic gas conditioning while preserving perfusion, temperature control, live readout compatibility, and chip accessibility.

2. Materials & Methods

2.1 Experimental setup

All experiments were performed using a VitroFlow.Bio v2.3 equipped with the Be-Flow Standard chip by Beonchip. The experimental workflow was adapted from the previously established VitroFlow.Bio validation protocol for microfluidic chip operation, including downstream flow sensing, dissolved oxygen monitoring, sterility assessment, and microscopy-based chip evaluation. Be-Flow chips, tubing, and fluidic components were prepared under sterile conditions before connection to the chip. The chip was connected under liquid-to-liquid contact after complete priming of the inlet line to minimize air introduction, followed by a short leak and bubble inspection before setting the final flow rate.

2.2 Dissolved oxygen measurements

Dissolved oxygen was measured downstream of the chip using an optical O₂-Probe by PyroScience. Unless stated otherwise, measurements were performed in the contamination-sensitive culture medium used in all validation experiments for sterility assessment. The VitroFlow.Bio incubator was heated to 37°C during all O₂ experiments. However, the temperature recorded by the PyroScience sensor reflects the downstream measurement position rather than the internal incubator temperature. Long-term incubation performance was assessed by perfusing the Be-Flow chip at 50 µL/min under 5% CO₂ for 72 h while continuously recording dissolved oxygen.

To compare gas-conditioning performance with a conventional incubator, the VitroFlow.Bio and

a conventional CO₂ incubator were sequentially set to 5%, 10%, and 20% CO₂. For each setpoint, measurements in the VitroFlow.Bio were recorded after the system had reached operational equilibrium. Measurements in the conventional incubator were taken after 30 min of incubation. In addition, the spatial variation of dissolved oxygen was assessed by measuring incubated liquids at four positions within the conventional incubator and comparing these measurements with those obtained using VitroFlow.Bio.

To determine the lower oxygen limit achievable with the VitroFlow.Bio, the system was subsequently operated at 100% CO₂ for 30 h while continuously recording dissolved oxygen concentrations.

2.3 Flow stability and sterility assessment

Flow performance was monitored throughout the 96 h experimental period using a Sensirion flow sensor to assess whether the target flow rates were maintained during prolonged operation.

Sterility was evaluated over the same experimental period using culture medium and extracellular acidification as an indicator of microbial contamination, analogous to the previous contamination assay.

2.4 Biological proof-of-principle experiment

For biological demonstration of reduced-oxygen conditions, *Rhynchomonas* sp. protozoa were cultured in the chips using WC medium. WC medium was used as a freshwater substitute containing defined macronutrients, trace elements, vitamins, and buffer components to adjust the pH to approximately 7.8–8.5. Nitrogen was used instead of compressed air to lower

Table 1: Overview of experimental conditions tested in the VitroFlow.Bio

Parameter	Experimental conditions
Flow rate	50µL/min & 100µL/min
Temperature	37°C, RT for protozoan cell culture
Gas	5%, 10%, 20% & 100% CO ₂ ; Cell culture 100% N ₂

oxygen availability while maintaining CO₂-based pH control and avoiding an abrupt pH shift. Protozoa were evaluated by abundance, morphology, and motility using an inverted microscope, thereby also assessing the optical accessibility of the Be-Flow chip during operation.

2.5 Data analysis

Raw data were processed using PyroScience DataInspector, Sensirion DataViewer, Microsoft Excel, and RStudio.

3. Results

3.1 Oxygen conditioning

The VitroFlow.Bio incubation module was evaluated during continuous perfusion of the Be-Flow Standard chip to assess oxygen conditioning, long-term stability, sterility, and biological compatibility under defined gas conditions.

During 72 h incubation at 5% CO₂, the dissolved oxygen concentration reached a stable plateau of approximately 7.300 µg/L after 6 h (Fig. 3A). This plateau was comparable to the oxygen concentration measured under standard CO₂ incubator conditions and remained within the calculated target range. After equilibration, c(O₂) remained stable throughout the entire measurement period, with fluctuations corresponding to changes in ambient temperature that affected the calculated gas solubility and consequently the sensor readout. These observations indicate stable gas conditioning during continuous perfusion over 72 h.

A direct comparison of c(O₂) at 5%, 10%, and 20% CO₂ showed that the values obtained with VitroFlow.Bio were comparable to those measured in a standard CO₂ incubator (Fig. 3B). In some conditions, the VitroFlow.Bio measurements were closer to the calculated target range than those obtained with the standard incubator. The comparison also showed that identical media should be used for all measurements, as medium composition strongly influences gas solubility and therefore affects the measured dissolved oxygen concentration. As shown in Fig. 3B, the variability of dissolved oxygen concentrations

was lower in the VitroFlow.Bio than in the standard incubator setup. The standard incubator exhibited greater spatial variation, whereas VitroFlow.Bio maintained a narrower distribution of c(O₂) values under the tested conditions.

To assess the lower oxygen limit of the system, the Be-Flow Standard setup was perfused under 100% CO₂. Following initiation of incubation, dissolved oxygen continuously decreased and approached zero after approximately 22.1 h (Fig. 3C). This corresponded to an overall oxygen depletion rate of -8.5 µg/min and a maximum depletion rate of -35.74 µg/min during the approximately linear phase of the depletion curve.

The apparent minimum c(O₂) of -8.5 µg/L does not represent a physically negative oxygen concentration but results from the measurement configuration. The cell-culture-relevant fluidic regions, including the CO₂ incubator and chip chamber, were maintained at 37°C, whereas the oxygen probe was positioned downstream of the chip. Consequently, the downstream temperature sensor recorded ambient rather than incubation temperature. As a result, the calculated oxygen solubility coefficient at the measurement position was higher than within the incubator, leading to an apparent negative value. Functionally, this measurement corresponds to dissolved oxygen concentrations approaching zero under the tested 100% CO₂ condition.

3.2 System stability

Peristaltic tubing performance was monitored as a secondary stability parameter. Tubing was exchanged on day 0, and flow rates were checked daily using internal and external flow sensors. Stable flow generation was maintained for up to 16 days before tubing wear and flow drift required replacement.

Sterility was monitored during two 120 h experiments using phenol red in the medium. Three of four channels remained contamination-free throughout the experiment. Contamination in one channel occurred following repeated disconnection and reconnection of the downstream oxygen probe under non-sterile conditions.

Setup

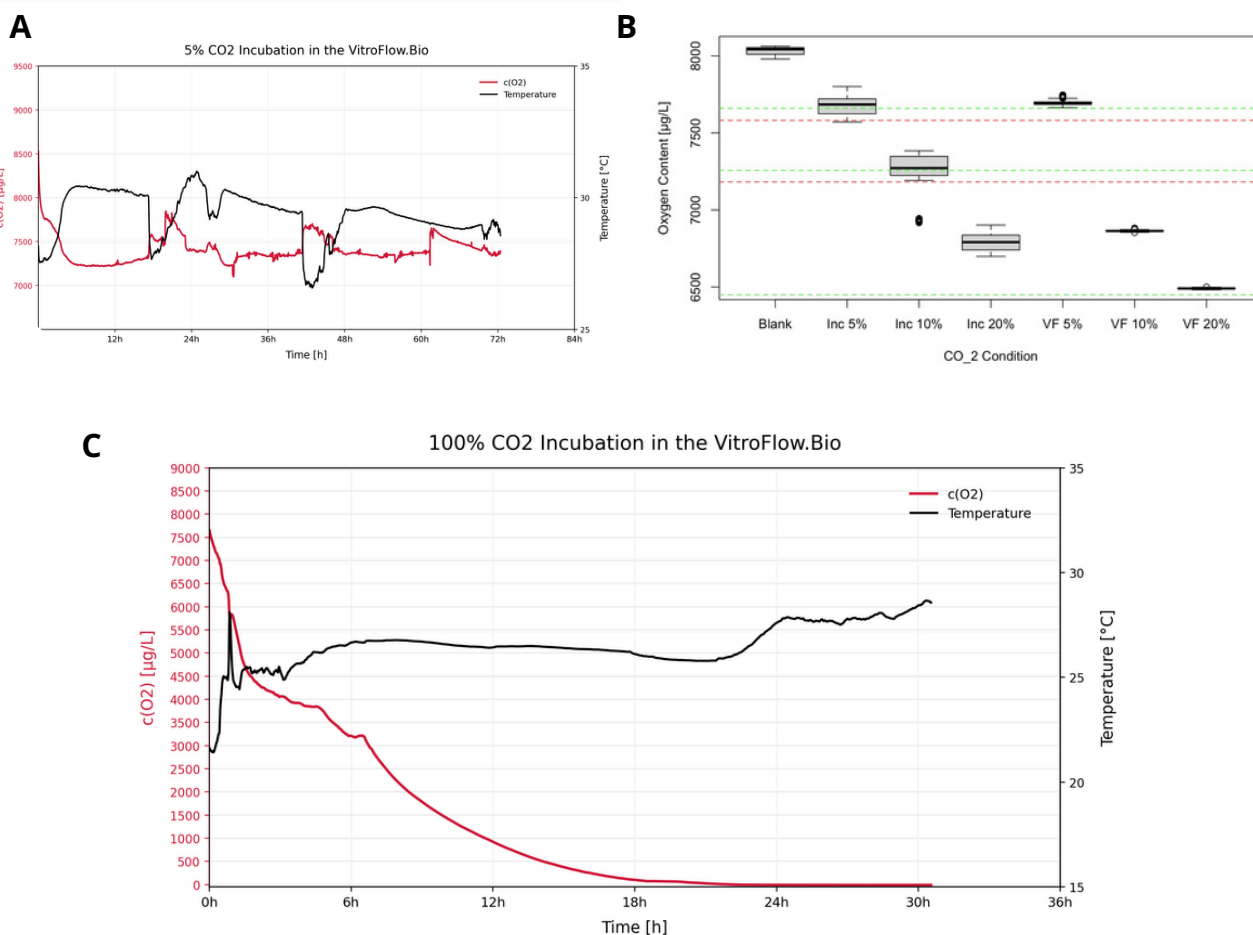
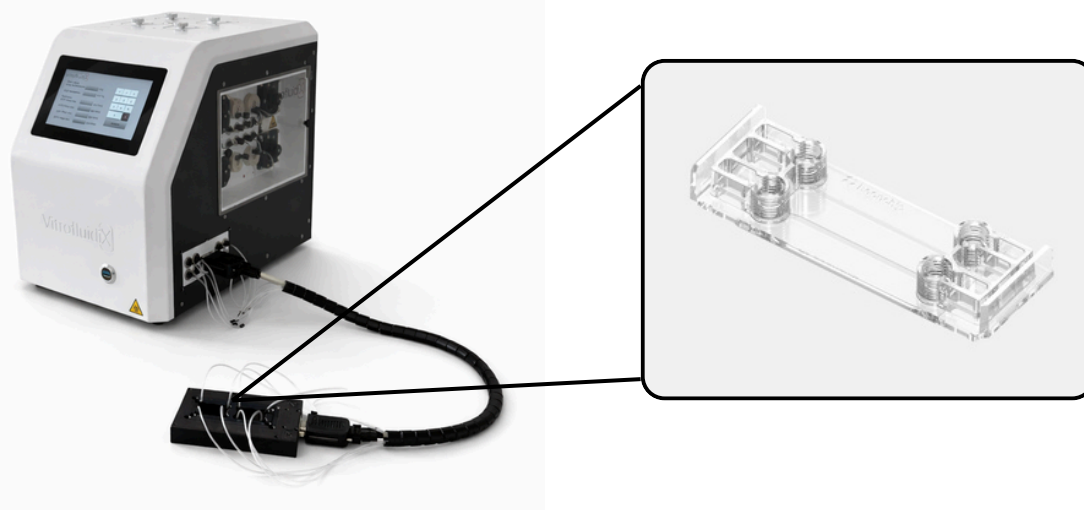


Figure 3: Seen in the photos labeled “Setup” the setup as combination of the VitroFlow.Bio in combination with the Be-Flow Standard of Beonchip is seen. **A** shows the measurement of O₂ concentration at 5% CO₂ incubation over 72h with the left y-axis indicating O₂-concentrations in µg/L and the right x-axis indicating the temperature in °C. Graph **B** shows a boxplot comparing the measured O₂ concentrations in µg/L in four corners of a standard incubator with four measurements of the VitroFlow.Bio and dashed lines indicating theoretical O₂ solubility. Graph **C** shows the measurement of O₂ concentration at 100% CO₂ incubation over 24h with the left y-axis indicating c(O₂) in µg/L and the right y-axis indicating the temperature in °C.

3.3 Biological proof-of-principle

In this preliminary qualitative proof-of-principle experiment, the Be-Flow Standard chip was operated in combination with VitroFlow.Bio under strongly oxygen-depleted conditions. Protozoa cultured under 100% N₂ perfusion at 100 µL/min remained visually viable (n = 5 on day 0; n = 6 on day 3), comparable to cultures maintained under ambient atmosphere (n = 20 on day 0; n = 22 on day 3). No obvious changes in morphology or motility were observed by microscopy. These observations are consistent with the reported ability of *Rhynchomonas* spp. to persist under both normoxic and anaerobic conditions.

4. Discussion

The results show that VitroFlow.Bio can establish and maintain defined gas conditions during continuous perfusion of the Be-Flow Standard chip. Incubation at 5% CO₂ is one of the most widely used conditions in mammalian cell culture, and the 72 h experiment demonstrated stable oxygen conditioning throughout the observation period. The long-term experiments presented in this application note were terminated according to the experimental design, and no technical indications were observed that would suggest that longer incubations cannot be performed under comparable conditions.

The comparison with a conventional CO₂ incubator showed that VitroFlow.Bio produced dissolved oxygen concentrations comparable to those measured at 5%, 10%, and 20% CO₂. These findings support its use as an integrated incubation and perfusion platform for microfluidic chips without requiring placement of the complete setup inside a conventional incubator. A key advantage is that the microfluidic chip remains accessible for microscopy during ongoing perfusion and incubation.

The variance analysis further suggests that VitroFlow.Bio provides more spatially uniform gas conditioning than a conventional incubator. Greater variability is expected in conventional incubators because gas inlet position, sensor location, chamber geometry, and the position of the cell culture vessel can all contribute to local

concentration differences. VitroFlow.Bio reduces this variability by conditioning the medium directly within the fluidic path. Nevertheless, deviations from calculated oxygen solubility remain possible and are likely influenced by factors such as ambient pressure, temperature, medium composition, and the fact that blank measurements were not performed simultaneously with every device measurement. Under 100% CO₂, the system reduced dissolved oxygen concentrations to near-zero levels after approximately 22.1 h and maintained this lower plateau thereafter. The total decrease in dissolved oxygen from the initial measurement to the plateau was approximately 7.680 µg/L, corresponding to an average depletion rate of approximately 8.5 µg/min and a maximum depletion rate of 35.74 µg/min. These results indicate that VitroFlow.Bio is capable of establishing gas conditions compatible with hypoxic as well as strongly oxygen-depleted microfluidic applications. As with all gas-conditioning experiments, sufficient equilibration time should be allowed before introducing sensitive cell cultures.

Peristaltic tubing remained stable for up to 16 days, supporting the recommended 14-day replacement interval. Sterility monitoring showed contamination in one of four channels. Because contamination occurred after repeated disconnection and reconnection of the oxygen probe under non-sterile environmental conditions, it is most likely attributable to experimental handling rather than continuous closed-system operation.

Previous VitroFlow-based cell culture studies have shown that human colorectal cancer cells can be cultured under continuous perfusion with stable temperature and gas supply (Altringer et al., 2026). The preliminary protozoa experiments presented here extend this application space by demonstrating visually maintained viability and unchanged morphology and motility under 100% N₂ perfusion at 100 µL/min. These observations suggest that VitroFlow.Bio may also support cultivation of oxygen-sensitive environmental samples without the use of oxygen scavengers while retaining live microscopy access during incubation.

5. Conclusion

The experiments presented in this application note show that VitroFlow.Bio can establish controlled gas environments ranging from conventional 5% CO₂ incubation to strongly oxygen-depleted conditions during continuous microfluidic perfusion. This makes the platform well suited for gas-controlled microfluidic cell culture and organ-on-a-chip applications while maintaining continuous perfusion, temperature control, and direct microscopic accessibility.

Further studies on anaerobic protozoa cultivation are planned to expand the biological validation of the platform. For hypoxic mammalian cell culture, VitroFlow.Bio can be equipped with an additional N₂ valve upon request, as CO₂-only hypoxia would result in excessive pH shifts in bicarbonate-buffered media. Alternatively, defined premixed gas mixtures can be supplied through the compressed-air inlet in the standard configuration.

The data presented in this application note will support further calibration of the system to improve agreement between measured dissolved oxygen concentrations and their calculated target values.

6. Sources

C. Altringer, P. Fuss, C. Kneer & D. P. R. Günter, (2026) A Dynamic Colorectal-Cancer-on-a-Chip Model Enabled by the VitroFlow System, www.vitrofluidix.com

M. Priya, A. Haridas & V. B. Manilal, 2008, "Anaerobic protozoa and their growth in biomethanation systems", Biodegradation, DOI 10.1007/s10532-007-9124-8

R. Battino & H. L. Clever, 1966, "Solubility of Gases in Water", Illinois Institute of Technology

R. Sander, 2023, "Compilation of Henry's law constants (version 5.0.0) for water as solvent", Jr. f. Atmospheric Chemistry and Physics, DOI 10.5194/acp-23-10901-2023

Beonchip, obtained 2026, "Be-Flow product guide", www.beonchip.com