

# A novel method for gas mixing and distribution in multi-chamber embryo incubators

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## Abstract.

**BACKGROUND:** High-quality control of the gas environment in incubators is crucial for *in vitro* embryo development, which requires high accuracy, fast recovery, and low gas consumption.

**OBJECTIVE:** In this study, we propose a novel gas mixing and distribution system and method as an alternative solution for multi-chamber embryo incubators.

**METHODS:** The system-based embryo incubator enables a controllable gas circulation process and a quantitative supply of CO<sub>2</sub> and N<sub>2</sub>. To determine the optimal parameters for the mixing time and flow rate of the circulated gases, we conducted contrast experiments on the system-based incubator. To evaluate the performance of the gas system in the incubator, we conducted tests under four different initial conditions, simulating various practical application scenarios. Furthermore, we performed a mouse embryo assay to assess the system's effectiveness.

**RESULTS:** The results show that the system achieved a gas concentration accuracy of  $\pm 0.2\%$  (volume fraction) after stabilization, a minimum recovery time of 5 minutes, an average consumption of 8.9 L/d for N<sub>2</sub> and 0.83 L/d for CO<sub>2</sub> during routine operation, and a blastocyst rate exceeding 90% observed after 96 hours of culture in the incubator.

**CONCLUSION:** The system and method demonstrate a significant advantage in terms of low gas consumption compared to existing incubators, while still maintaining high accuracy and fast recovery.

**Keywords:** Gas mixing, gas distribution, concentration control, low gas consumption, embryo incubator

## 1. Introduction

Regulating the media, temperature, humidity, and gas mixture in the *in vitro* biochemical environment is crucial for cell growth and development. Among these factors, the gas mixture plays a significant role in maintaining the pH of the media at an appropriate level [1]. Generally, most cells should be cultured in a 5% CO<sub>2</sub> and air environment [2]. However, embryos grow best under a gas environment

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containing 6% CO<sub>2</sub>, 5% O<sub>2</sub>, and 89% N<sub>2</sub> as widely reported. Several studies have shown that a low oxygen concentration of 5% can enhance oocyte maturation and blastocyst development, achieving a higher potential for ongoing clinical pregnancy [3,4,5,6].

Conventional water jacket incubators (Thermo Fisher Forma 3110, ASTEC WMI-165) are originally designed for large-scale somatic cell culture and have an internal volume of about 180 L. These incubators use CO<sub>2</sub> and O<sub>2</sub> sensors to measure the concentrations of mixed gases and employ a fan to promote mixing to achieve the desired concentrations. However, when the door is closed, it takes more than 120 minutes for these incubators to recover the gas concentrations, resulting in high gas consumption [7]. As an alternative technology, multi-chamber embryo incubators have gained popularity in IVF (*in vitro* fertilization) laboratories. These incubators have superior temperature and gas control capabilities [7,8,9]. The individual chamber design of these incubators offers significant advantages over conventional methods in terms of temperature and gas recovery. Due to their smaller interior volume, the temperature and gas levels in these incubators typically reach equilibrium within one minute and three minutes, respectively, after any disturbance, and then stabilize at the setpoint [7].

Multi-chamber benchtop incubators can be categorized into two types based on the gas supply mode [10]: one is supplied with a medical grade pre-mixed gas containing 6% CO<sub>2</sub>, 5% O<sub>2</sub>, and 89% N<sub>2</sub> (Genea Biomedex Geri) [11], while the other is supplied with pure CO<sub>2</sub> and pure N<sub>2</sub> (ESCO Medical Miri TL) [12]. In the former case, the gas is released into each individual chamber through a solenoid valve and then vented directly to the atmosphere, leading to higher gas consumption compared to the circulation system. Additionally, the stability of gas concentration in the cylinder is influenced by various factors, including ambient temperature. In the latter case, an air pump is used to drive the gas, achieving mixing and distribution through circulation. The automatic program enables the regulation of gas to reach any desired setpoint, typically ranging from 3–10% for CO<sub>2</sub> and 5–20% for O<sub>2</sub>, to accommodate different culture requirements. The gas consumption under normal conditions, as per the standard operating procedure, is less than 2 L/h for CO<sub>2</sub> and 5 L/h for N<sub>2</sub>, which is considered suboptimal. Moreover, using a single air pump, the incubator cannot guarantee uniformity of gas across all chambers due to the flow resistance differences between channels. Therefore, further optimization of existing gas systems in multi-chamber incubators is necessary to improve concentration accuracy, recovery time, environmental uniformity, gas consumption, gas purification, and minimize disturbance to embryos.

To explore a better solution for addressing the limitations of the gas system in a multi-chamber incubator, we propose a novel gas mixing and distribution system, along with a theoretical method for calculating the inflow volume of the two supply gases. Through conducting experiments and optimizing the system parameters, we demonstrate that the system-based incubator achieves high accuracy and fast recovery, while also significantly reducing gas consumption. The system and method presented in this study can serve as a valuable reference for the development of multi-channel gas distribution systems in various applications.

## 2. Materials and methods

### 2.1. Gas mixing and distribution system

The proposed system of gas mixing and distribution is illustrated in Fig. 1. Two MFCs are used to accurately regulate the flow rate and volume of CO<sub>2</sub> and N<sub>2</sub> from the cylinders to the gas mixer [13,14]. The gas mixer is used to achieve gas mixing within the system [15,16]. The system consists of twelve individual chambers, each connected to a micro air pump and a check valve. The air pumps are used to

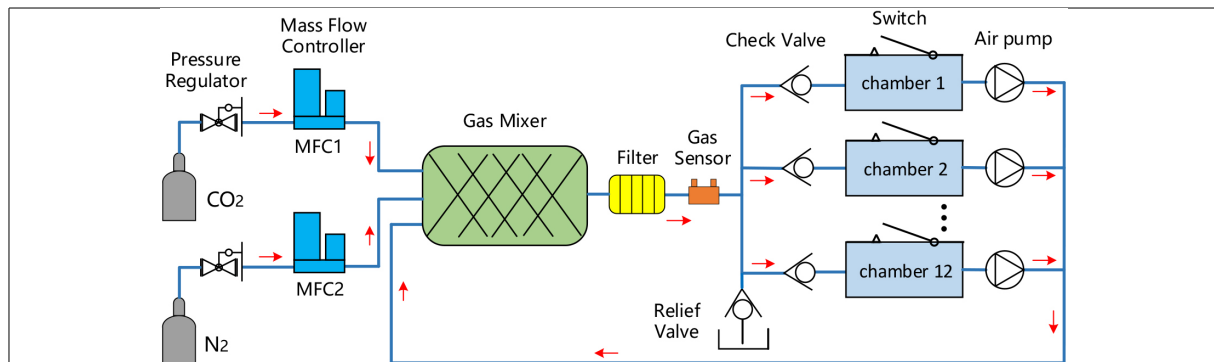


Fig. 1. Schematic of the gas mixing and distribution system for a multi-chamber embryo incubator. The red arrows indicate the gas flow direction.

drive gas flow and circulation in the system, as well as distribute gas to the chambers. The check valve prevents air interference when a chamber lid is opened. The state of each chamber lid (open or closed) can be identified by a switch sensor. The gas, propelled by the air pumps, flows from the mixer into the chambers and then returns back to the mixer to achieve circulation, as indicated by the red arrows in Fig. 1. The air pump is automatically deactivated when chamber lid is open and only operates when the chamber lid is closed. Consequently, the gas circulation part remains enclosed as long as at least one chamber lid is closed. The gas sensor is positioned downstream of the gas mixer and upstream of the chambers, along the flow direction, to measure the gas concentrations (volume fraction). Additionally, a relief valve, positioned between the gas sensor and the check valves, serves as a crucial component. Its purpose is to release any excess gas when the internal pressure exceeds atmospheric pressure.

The standard operation process of the system starting from the initial gas condition consists of the following four stages:

- (1) All the air pumps blow at a high flow rate for several minutes, promoting rapid gas flow and mixing through the gas mixer. This process also ensures the distribution of gas throughout the chamber. Mixing is performed to minimize the gas concentration gradient and achieve a consistent gas concentration throughout the system. The effect of gas mixing over time can be evaluated by measuring the concentrations.
- (2) If the measured gas concentrations after mixing fall outside the acceptable range, the MFCs will control the flow of  $\text{CO}_2$  and  $\text{N}_2$  separately into the gas mixer at specific volumes. At the same time, the original gas in the mixer is slowly discharged through the relief valve until the MFCs are closed. It is important to note that a small quantity of new gas may also escape during this process. The inflow volume of  $\text{CO}_2$  and  $\text{N}_2$  can be calculated based on system parameters and measured gas concentrations. Additionally, it is crucial to keep the air pumps closed during this stage to ensure thorough replacement of the original gas with the new gas.
- (3) Similar to stage 1, all the air pumps are activated to blow for several minutes, to facilitate the mixing of the new gas with any remaining original gas. The goal is to achieve a high level of uniformity in gas concentration throughout the entire system.
- (4) All the air pumps are set to a low flow rate, known as breezing, to prevent air leakage into the chamber and potential impact on cell development. This breezing state continues as long as the lid remains closed, unless there is a change in gas concentration. When the lid is opened and closed again, a blowing operation is initiated instead of breezing, allowing the system to quickly return to the previous level within a short period, usually within three minutes.

In summary, the system raises two main concerns: determining the optimal mixing parameters and calculating the inflow volume of CO<sub>2</sub> and N<sub>2</sub>.

## 2.2. Calculation of inflow volume

The method for calculating the theoretical inflow volume of CO<sub>2</sub> or N<sub>2</sub> passing through the MFC can be derived using the following approach. For a single gas in the circulation part, the calculation involves adding the volume of the original gas to the volume of the newly introduced gas and then subtracting the volume of the gas discharged from the relief valve. This resulting volume should be equal to the product of the total volume of the circulation part and the target concentration. Therefore, the calculation for the inflow volume of CO<sub>2</sub> or N<sub>2</sub> passing through the MFC can be represented as:

$$(\text{Volume of original gas} + \text{Volume of new gas} - \text{Volume of discharged gas}) = (\text{Total volume of circulation part}) \times (\text{Target concentration}).$$

Consequently, the inflow volume of CO<sub>2</sub> and N<sub>2</sub> satisfies the following equations, respectively:

$$VC_c + X - (X + Y)C_c = VC_{ct} \quad (1)$$

$$VC_o - (X + Y)C_o = VC_{ot} \quad (2)$$

where V is the total volume of the circulation part, C<sub>c</sub> and C<sub>o</sub> are the current concentrations of CO<sub>2</sub> and O<sub>2</sub>, X and Y are the inflow volume of CO<sub>2</sub> and N<sub>2</sub>, C<sub>ct</sub> and C<sub>ot</sub> are the target concentrations of CO<sub>2</sub> and O<sub>2</sub>, respectively. The gas concentrations are expressed by volume fraction.

Thus, the X and Y can be calculated as follows:

$$X = \frac{V(C_o C_{ct} - C_c C_{ot})}{C_o} \quad (3)$$

$$Y = \frac{V(C_o - C_{ot} + C_c C_{ot} - C_o C_{ct})}{C_o} \quad (4)$$

When X and Y are both positive, the result indicates a feasible solution that the target concentrations of CO<sub>2</sub> and O<sub>2</sub> can be achieved by injecting the corresponding volume of CO<sub>2</sub> and N<sub>2</sub>. Otherwise, there are no feasible solutions, which results from the reason C<sub>o</sub> < C<sub>ot</sub>, revealing insufficient volume of O<sub>2</sub> within the system. In such situation, opening chamber lids or waiting for air leakage could increase the O<sub>2</sub> level in practice. Furthermore, in cases where O<sub>2</sub> could not be supplied in a short time, owing to the greater significance of CO<sub>2</sub> for cell development, the CO<sub>2</sub> concentration should be ensured to reach the target value preferentially regardless of O<sub>2</sub>. However, there exist two cases that C<sub>c</sub> > C<sub>ct</sub> and C<sub>c</sub> < C<sub>ct</sub> when C<sub>o</sub> < C<sub>ot</sub> occurs:

(1) For C<sub>c</sub> > C<sub>ct</sub>, N<sub>2</sub> is required to dilute CO<sub>2</sub> and the added volume satisfies the following equation:

$$VC_c - YC_c = VC_{ct} \quad (5)$$

The new Y can be calculated as follows:

$$Y = \frac{V(C_c - C_{ct})}{C_c} \quad (6)$$

(2) For C<sub>c</sub> < C<sub>ct</sub>, more CO<sub>2</sub> is required and the added volume satisfies the following equation:

$$X + VC_c - XC_c = VC_{ct} \quad (7)$$

The new X can be calculated as follows:

$$X = \frac{V(C_{ct} - C_c)}{1 - C_c} \quad (8)$$

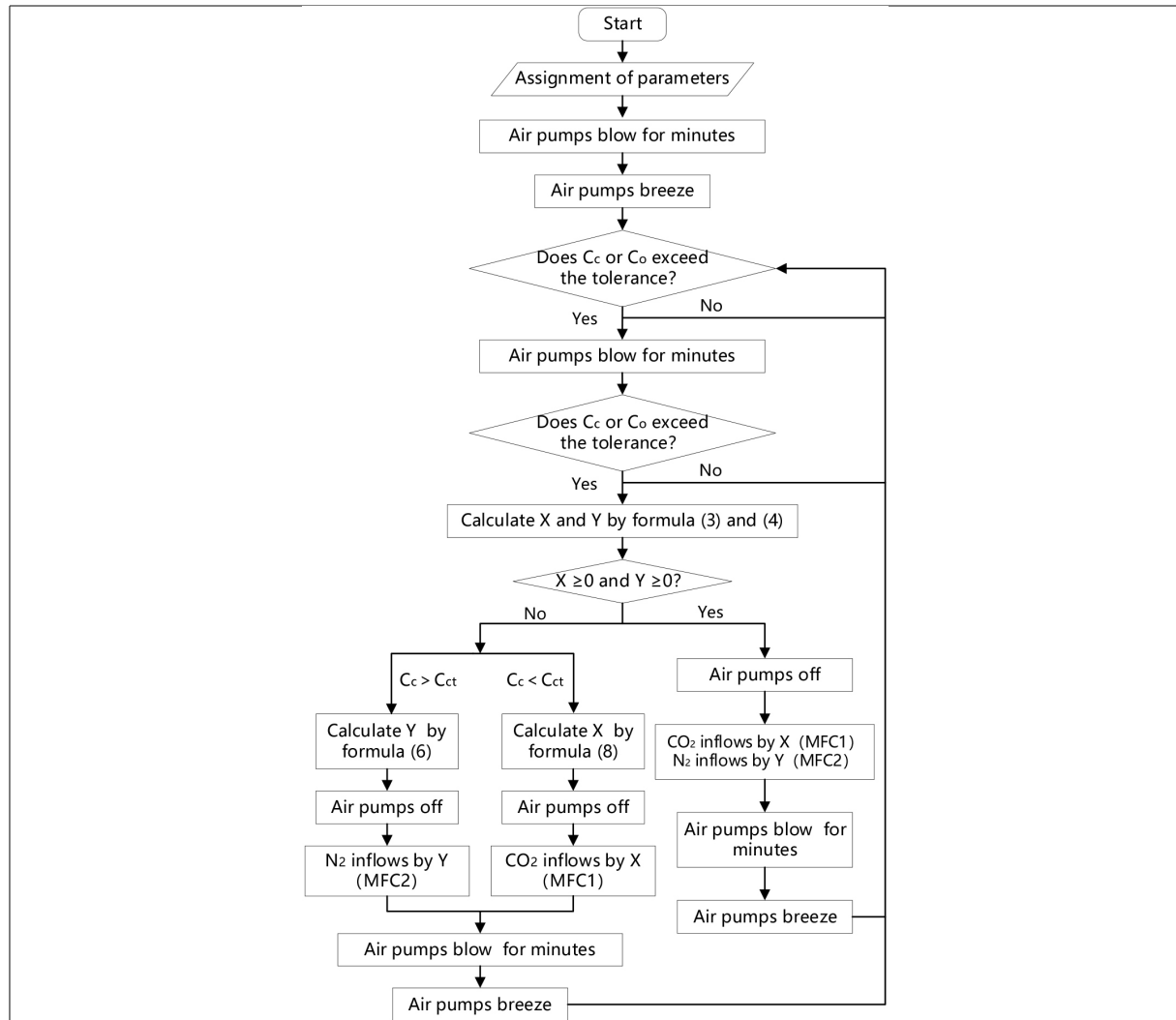


Fig. 2. Flow chart of gas mixing and distribution system.

The workflow of the gas mixing and distribution system, along with the operation process and calculation method of inflow volume, is illustrated in Fig. 2. This workflow serves as the basis for developing an automatic control program for a multi-chamber incubator.

### 2.3. System-based incubator and experiment

A system-based multi-chamber time-lapse embryo incubator was assembled based on the fabricated components, as shown in Fig. 3. The incubator was a compact device containing 12 chambers arranged in two rows (Fig. 3a). The gas system components were mainly installed at the rear of the incubator, including air pumps (Thomas Diaphragm pump, model 2002 VD BLDC, Monroe, USA), CO<sub>2</sub> sensor (Novasis Innovazione Novagas2, model NG2-A-2, Italia), O<sub>2</sub> sensor (SST Sensing LuminOx, model LOX-02-F, Coatbridge, UK), MFCs (AITOLY, model MFC300\_RS485, Wuxi, China), gas mixer (Henan

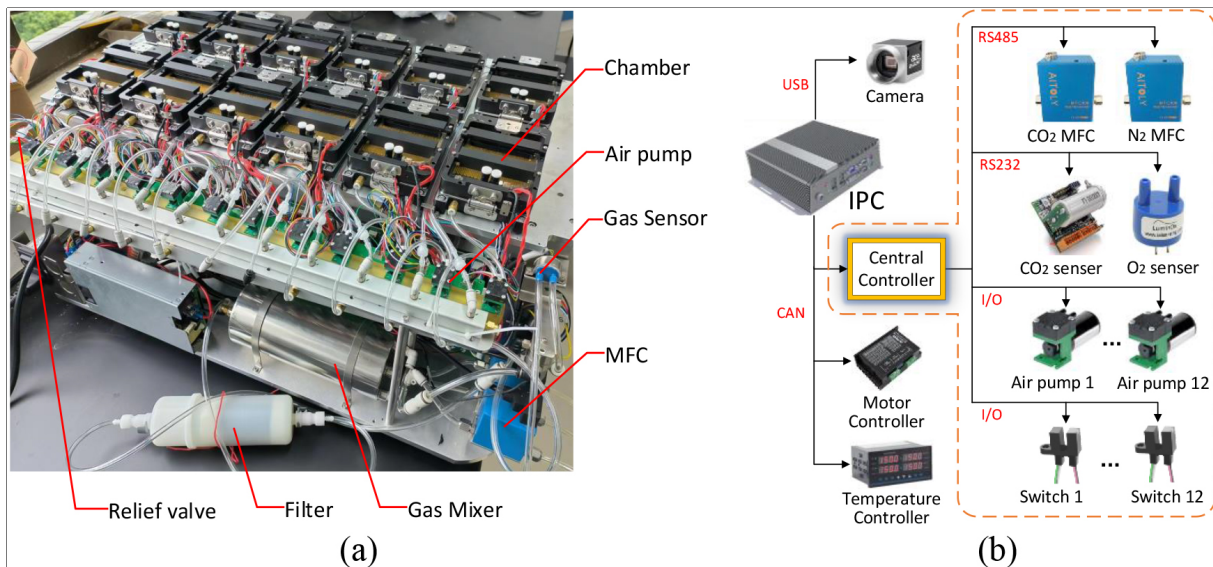


Fig. 3. A system-based multi-chamber time-lapse embryo incubator. (a) Layout of the relevant components of the gas system. (b) The control structure of the incubator. The gas mixing and distribution system is circled with a dashed line.

Xingchao Electromechanical Equipment Co., Ltd., China), relief valve (SMC, model AKH06, Japan), and filter (Hangzhou Cobetter Filtration Equipment Co., Ltd., China). The maximum flow rate of MFC for N<sub>2</sub> and CO<sub>2</sub> are 500 sccm and 50 sccm, respectively. The gas mixer was designed as an enclosed thin-walled cylinder, filled with three SV static mixing units and each positioned at a 90-degree angle to the adjacent units. The total volume of the gas circulation part was 2000 mL, with 950 mL for the gas mixer and 1050 mL for other components (including the chambers). The gas central controller plays a crucial role in the device, which also includes motor controllers, temperature controllers, and a camera (Fig. 3b).

The experiment of gas mixing efficiency was performed on the incubator. Firstly, the gas mixer was disconnected from the circulation part at the filter, making it open to the atmosphere. Secondly, CO<sub>2</sub> and N<sub>2</sub> were simultaneously introduced into the mixer at flow rates of 50 mL/min and 425 mL/min, respectively. This process lasted for more than 5 minutes to ensure that the gases were injected in a proportional ratio of 100 mL and 850 mL. It is important to note that the rest of the system was filled with air except for the mixer. Subsequently, the gas mixer was reconnected to the circulation part, and all the air pumps were activated to start operation. The concentrations of CO<sub>2</sub> and O<sub>2</sub> were then measured over time, expected to reach 5% and 11%, respectively, after stabilization.

In addition, to validate the performance of the proposed system and method, experiments were conducted under four initial conditions and routine operations. Instead of introducing external gas with a given concentration, an artificial offset method was used to simulate the conditions where CO<sub>2</sub> or O<sub>2</sub> concentration deviates from the target value.

#### 2.4. Mouse embryo assay

All animal procedures were approved by the Experimental Animal Welfare Ethics Committee of Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences. Six- to 8-week-old B6D2F1 female mice were administered 5 IU pregnant mare serum gonadotrophin (PMSG; Sigma), followed by 5 IU human chorionic gonadotrophin (hCG; Sigma) 48 h later. Females were then placed with

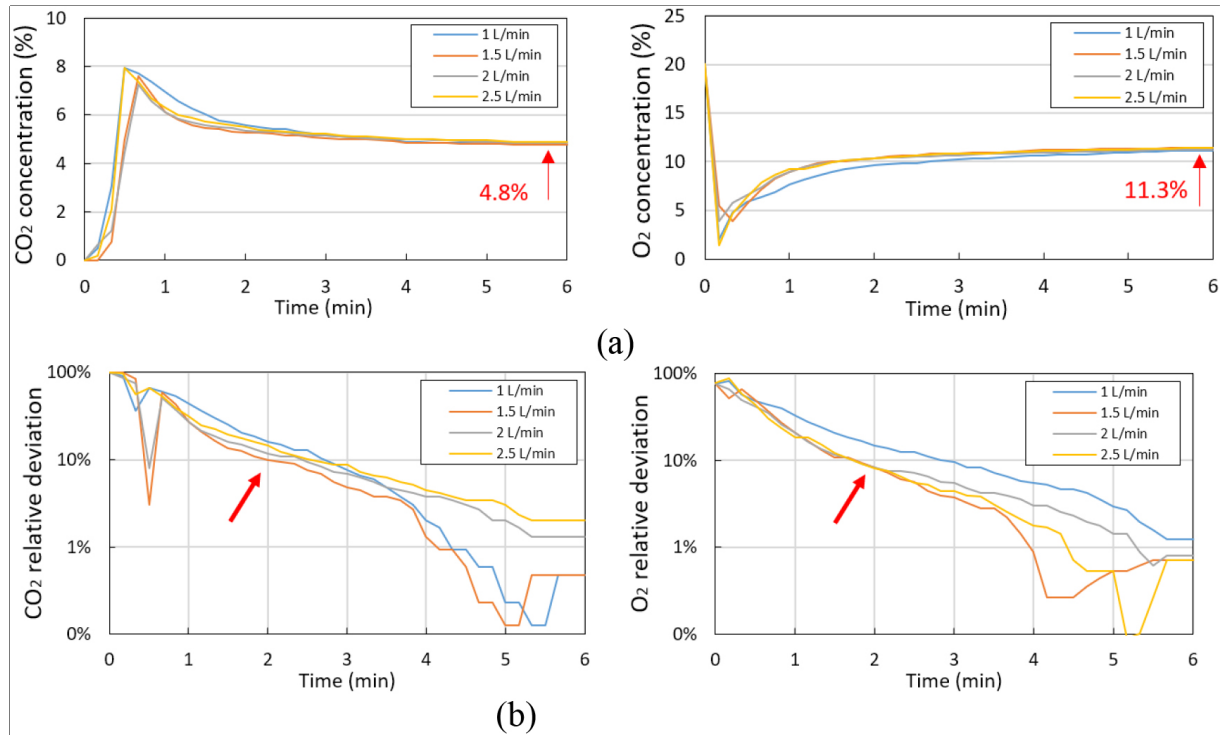


Fig. 4. Gas concentration measured under different flow rates. (a) Gas concentration changing over time after starting mixing. (b) Relative deviation of gas concentration to the steady state. The vertical coordinates are displayed in a logarithms scale.

adult B6D2F1 males of known fertility overnight and examined the following morning for vaginal plugs indicating that mating had occurred. Zygotes were collected at 21 h post-HCG in M2 (Nanjing Aibei) and denuded by a brief (less than 1 min) incubation with hyaluronidase (0.5 mg/ml, Sigma). Embryos were washed in M2 and once in the G1 (Vitrolife) culture medium, followed by random distribution of 14–16 zygotes into 50  $\mu$ l droplet of G1 culture medium overlaid with mineral oil (Dewin Medical) in 35 mm culture dishes. All embryos were cultured in G1 to the 8-cell stage, followed by culture for a further 48 h in G2 to the blastocyst stage. Subsequently, embryo cultures were performed in the system-based incubator with the target gas concentrations for CO<sub>2</sub> and O<sub>2</sub> set to 6% and 5% respectively, and the temperature set to 37°C. Embryo development was assessed by blastocyst rate after 96 hours of culture.

### 3. Results

#### 3.1. Gas mixing efficiency by circulation

The mixing efficiency by circulation is assessed based on the gas concentration over time. Concentration measurement is conducted under four different flow rates, ranging from 1 L/min to 2.5 L/min, with increments of 0.5 L/min. Before each test, the circulation part is filled with 100 mL of CO<sub>2</sub> and 850 mL of N<sub>2</sub>, while the other part contains 1050 mL of air. The concentrations of CO<sub>2</sub> and O<sub>2</sub> are recorded simultaneously every 10 seconds from the onset to reaching stability. The test lasts for 6 minutes (Fig. 4a). The expected values for CO<sub>2</sub> and O<sub>2</sub> are 5% and 11%, respectively. The final concentrations reach 4.8%

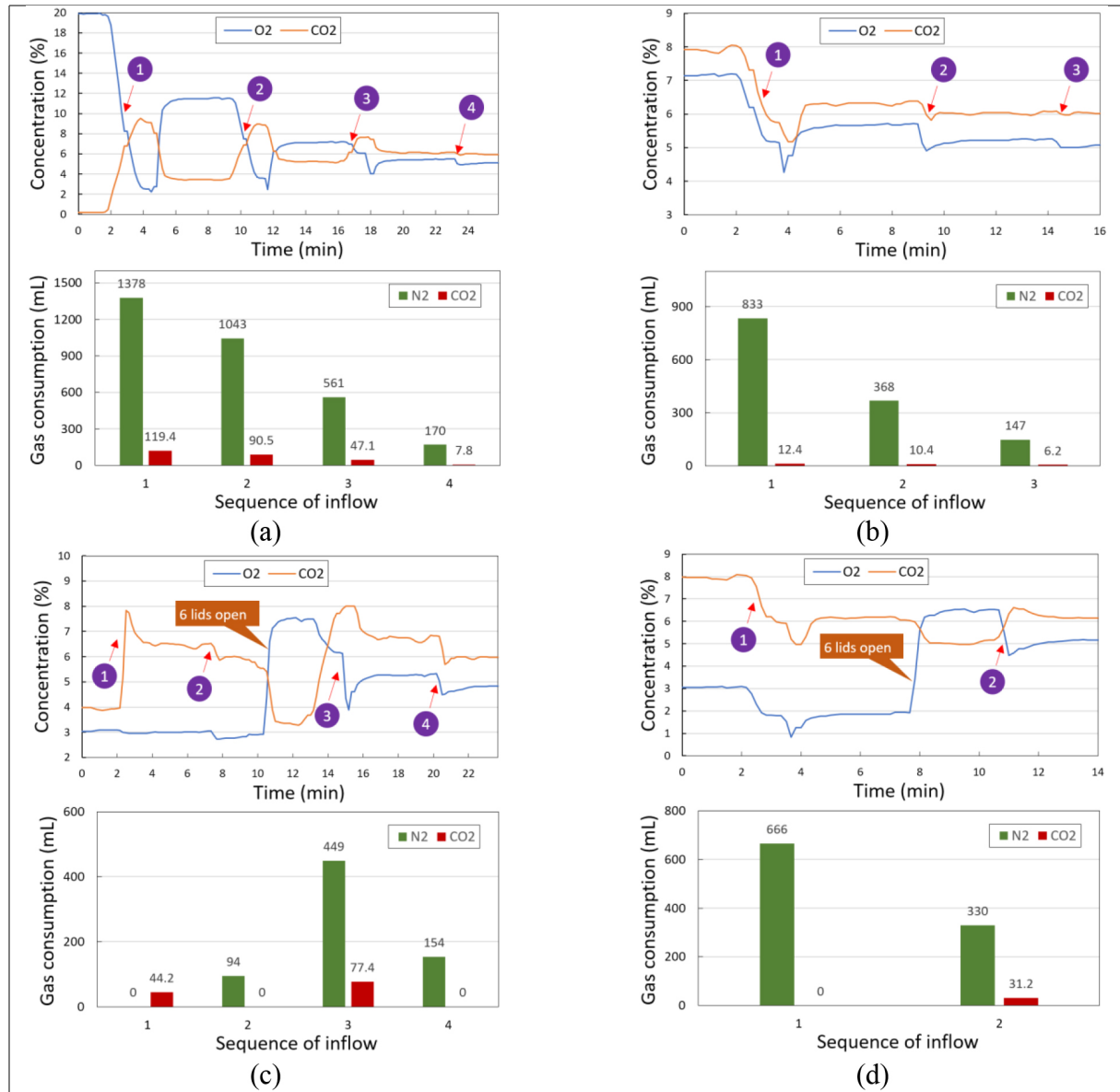


Fig. 5. Gas concentrations of CO<sub>2</sub> and O<sub>2</sub> and gas consumption of N<sub>2</sub> and CO<sub>2</sub> under the following four initial conditions: (a) CO<sub>2</sub> is lower and O<sub>2</sub> is higher (0% CO<sub>2</sub> and 20% O<sub>2</sub>). (b) Both of CO<sub>2</sub> and O<sub>2</sub> are higher (8% CO<sub>2</sub> and 7% O<sub>2</sub>). (c) Both of CO<sub>2</sub> and O<sub>2</sub> are lower (4% CO<sub>2</sub> and 3% O<sub>2</sub>). (d) CO<sub>2</sub> is higher and O<sub>2</sub> is lower (8% CO<sub>2</sub> and 3% O<sub>2</sub>). The sequence numbers of inflow at specific times are indicated by arrows and the manual interventions of chamber lids are indicated by text boxes.

and 11.3%, satisfying the measurement error. The relative deviation of the two gases from the steady state values is present in Fig. 4b. The optimal mixing time and flow rate for the system are 2 minutes and 1.5 L/min as indicated by the arrows in Fig. 4b, respectively, with  $\pm 10\%$  as the acceptance criteria.

### 3.2. Gas concentration and consumption

The performance of the system was tested according to the workflow illustrated in Fig. 2, under four



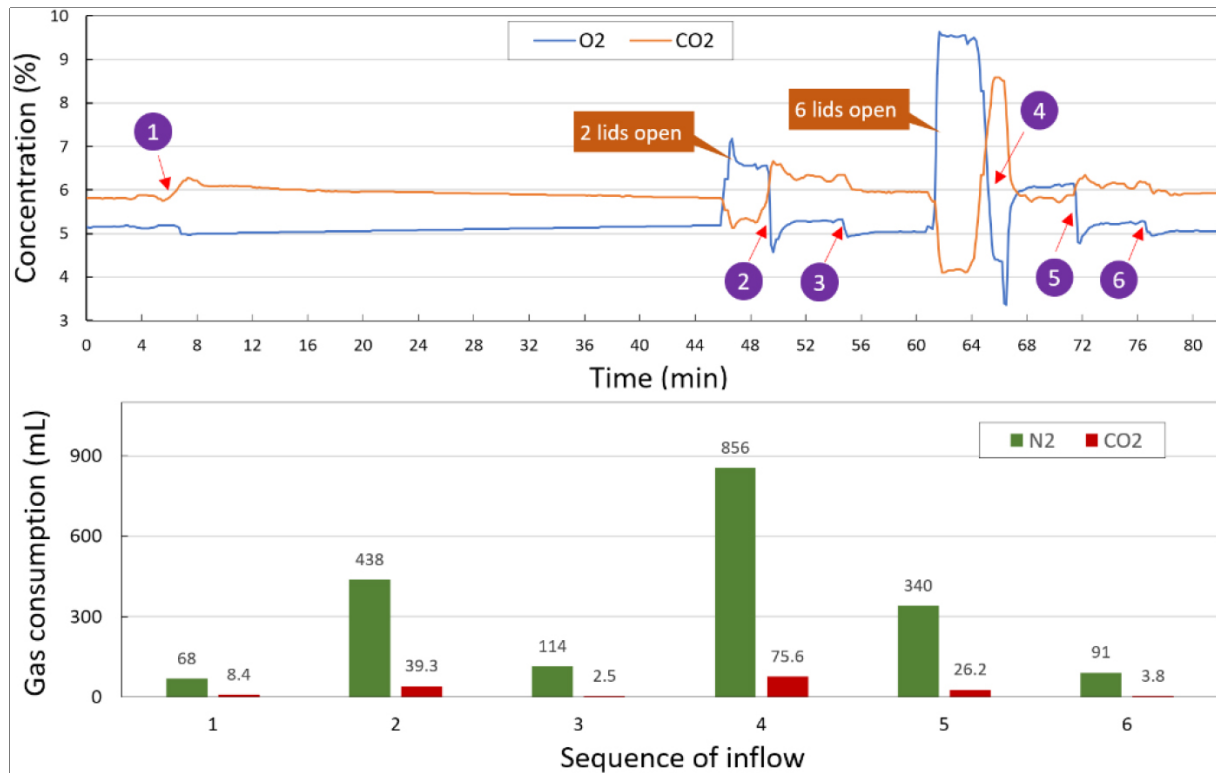


Fig. 6. Gas concentrations of CO<sub>2</sub> and O<sub>2</sub> and gas consumption of N<sub>2</sub> and CO<sub>2</sub> under routine operations.

initial conditions that cover common application scenarios encountered in practice. Instead of introducing external gases with specific concentrations, artificial offsets were applied by the software to generate the initial deviations, except for air. The target gas concentrations were set to 6% for CO<sub>2</sub> and 5% for O<sub>2</sub>, the best parameters for embryo development, based on which the artificial offsets were implemented at the beginning. The initial concentration combinations of the two gases are as follows: 0% CO<sub>2</sub> and 20% O<sub>2</sub>, 8% CO<sub>2</sub> and 7% O<sub>2</sub>, 4% CO<sub>2</sub> and 3% O<sub>2</sub>, and 8% CO<sub>2</sub> and 3% O<sub>2</sub>. These initial concentrations have been stabilized in preparation for subsequent testing. In addition, the acceptable absolute deviation from the target values is defined  $\pm 0.2\%$  for both gases. The change of gas concentration and consumption over time for the four conditions are described in Fig. 5. Furthermore, gas concentration and consumption were measured under routine operations, as shown in Fig. 6.

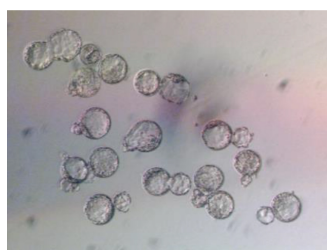
Figure 5a and b show that under the two initial conditions (0% CO<sub>2</sub> and 20% O<sub>2</sub>, 8% CO<sub>2</sub> and 7% O<sub>2</sub>), the concentrations of CO<sub>2</sub> and O<sub>2</sub> both stabilize in an acceptable range of  $\pm 0.2\%$  after several adjustments. Besides, under the two conditions (4% CO<sub>2</sub> and 3% O<sub>2</sub>, 8% CO<sub>2</sub> and 3% O<sub>2</sub>) in Fig. 5c and d, only CO<sub>2</sub> concentration reaches the target value while O<sub>2</sub> concentration decreases compared to the initial value. However, by introducing air into the system through the opening six lids, the O<sub>2</sub> is supplied and gas concentration eventually falls within the acceptable range.

When the gas concentration exceeds the acceptable range, the single adjustment time of the system is determined by the gas inflow volume, which equals the gas consumption (Fig. 5). Once all lids are closed and gas stabilization is achieved, the recovery time will not exceed 5 minutes after a new deviation occurs (Fig. 6).

In the scenario where the system is initially filled with air (0% CO<sub>2</sub> and 20% O<sub>2</sub>), the gas consumption

Table 1  
Mouse embryo development results after 96 hours of culture

Chamber	1	2	3	4	5	6
No. of embryos	15	16	15	15	16	14
No. of blastocysts	14	16	15	14	15	14
Blastocyst rate	93.3%	100%	100%	93.3%	93.7%	100%
Chamber	7	8	9	10	11	12
No. of embryos	15	16	14	15	14	15
No. of blastocysts	14	15	13	15	14	14
Blastocyst rate	93.3%	93.7%	92.8%	100%	100%	93.3%



(a)



(b)

Fig. 7. Morphology of mouse embryos cultured for 96 hours from zygotes in chamber 3 (a) and chamber 11 (b) of the incubator.

is significantly higher than the other three conditions, with a total volume of 3152 mL for N<sub>2</sub> and 263.8 mL for CO<sub>2</sub> (Fig. 5a). After stabilization, the gas system adjusts every 40 minutes with a typical consumption of 68 mL for N<sub>2</sub> and 8.4 mL for CO<sub>2</sub> (Fig. 6). Opening and closing two lids after stabilization, a consumption of 552 mL for N<sub>2</sub> and 41.8 mL for CO<sub>2</sub> is needed. Also, opening and closing six lids after stabilization, a consumption of 1287 mL for N<sub>2</sub> and 105.6 mL for CO<sub>2</sub> is needed. Consequently, in routine operations with five lids open per day, each time with six lids, the theoretical average gas consumption of N<sub>2</sub> and CO<sub>2</sub> per day can be calculated using the following formulas respectively:  $(24 \text{ h} \times 60 \text{ min}/40 \text{ min}) \times 68 \text{ mL} + 1287 \text{ mL} \times 5$ , and  $(24 \text{ h} \times 60 \text{ min}/40 \text{ min}) \times 8.4 \text{ mL} + 105.6 \text{ mL} \times 5$ . As a result, the gas consumption is estimated to be 8.9 L/d for N<sub>2</sub> and 0.83 L/d for CO<sub>2</sub>, indicating a remarkably low level.

### 3.3. Mouse embryo development

To evaluate the effect of the system-based incubator on embryo development, we performed a thorough mouse embryo assay that encompassed all chambers. The morphology of mouse embryos cultured from zygotes for 96 hours is illustrated in Fig. 7, and the corresponding statistical data is presented in Table 1. After 96 hours of culture, the percentage of mouse embryos reaching the blastocyst stage exceeded 90% in all chambers, with some reaching 100%. The results provide compelling evidence that the gas environment established in the incubator plays a crucial role in facilitating the successful development of embryos.

## 4. Discussion

Compared to the existing incubators, our system has 12 miniature air pumps for gas mixing and distribution, rather than a single air pump. This enables independent control of gas in each chamber,

231 ensuring that others still normally receive the gas supply when some chamber lids are opened. Moreover,  
232 this design ensures that the mixing gas is evenly distributed to each chamber and is not affected by the  
233 flow resistance differences between channels. Thus, our system promotes the establishment of a highly  
234 uniform gas concentration throughout the entire zone.

235 For the determination of mixing parameters, shorter mixing times and lower flow rates are two key  
236 design criteria. A shorter mixing time allows for faster system recovery, which enables rapid correction of  
237 gas concentrations when deviations occur. Air pump with a lower gas flow rate helps to reduce vibration  
238 and noise and minimize disturbance to the cells. A lower flow rate guarantees less cold gas into the  
239 chambers per unit time, minimizing the effect on the temperature distribution inside the chambers. In our  
240 system, both CO<sub>2</sub> and O<sub>2</sub> concentrations take 6 minutes to stabilize under different flow rates (Fig. 4a),  
241 which may be a little long for practical applications. Considering a relative deviation of  $\pm 10\%$  from  
242 the steady-state value as the acceptance criteria, we conclude that the optimal mixing time is 2 minutes  
243 and the optimal flow rate is 1.5 L/min (Fig. 4b). Further, the reason why neither higher nor lower flow  
244 rates are appropriate for gas mixing is due to the complex coupling problems of fluid mechanics and  
245 convection-diffusion of substances [16,17,18]. In this work, an SV type of static mixer is chosen over  
246 SX and SK types. The mixing effect of a static mixer depends on various factors, including the internal  
247 pressure, flow rate, the type of disturbance element, and the intake direction of the diversion element.  
248 Therefore, optimizing the design of the mixer to improve the mixing efficiency will be our future work.

249 To achieve stable gas concentration, multiple adjustments are required rather than just one, which can  
250 be attributed to two main reasons. First, in some cases, the inflow volume exceeds the interior volume  
251 of the mixer, causing a portion of the new gas to leak out through the relief valve as well. Second, the  
252 mixing time of two minutes determined to ensure a relative deviation of less than  $\pm 10\%$  under various  
253 initial conditions may not be sufficient for complete mixing. Nevertheless, despite these challenges, a  
254 gradual improvement in reducing gas concentration deviations is observed after several adjustments.  
255 For each subsequent adjustment, the gas concentrations of the system will be closer to the target value,  
256 and thereafter, the interval of stabilization between adjustments depends on the acceptable deviation.  
257 Note that there is a special case where the O<sub>2</sub> concentration is below the target value, which makes it  
258 theoretically impossible for the system to reach equilibrium. In this case, opening the chamber lids can  
259 effectively supply O<sub>2</sub>. Otherwise, it will take a considerable amount of time to wait for air to leak into the  
260 chambers to reach equilibrium. Generally, the O<sub>2</sub> concentration is not below the target value, except in  
261 specific situations, like calibrating sensors [19].

262 Sealing is a critical factor in ensuring the proper functioning of the gas system. In this regard, the  
263 chambers have a greater impact than the pipes, as their quality control procedures are more difficult than  
264 the latter. The air pump will operate at a low flow rate when the gas concentration is within an acceptable  
265 range. Meanwhile, the air could be gradually introduced to the gas system due to the negative pressure in  
266 the chambers. Poor sealing leads to an amount of air entering the system, resulting in longer adjustment  
267 time and even failure to reach the target value. Therefore, better sealing helps to maintain longer stability.  
268 In addition, introducing an appropriate amount of air is beneficial so that fresh gases of CO<sub>2</sub> and N<sub>2</sub> can  
269 be supplied during adjustments when gas concentrations deviate.

270 The blastocyst rate is a crucial indicator for evaluating the culture environment in mouse embryo  
271 assays. A blastocyst rate higher than 80% is generally considered acceptable. The results reveal that the  
272 blastocyst rate in all chambers exceeds 90%, with some reaching 100% (Table 1). It suggests that the  
273 device's condition is suitable for the development of mammalian embryos. However, it is important to note  
274 that the development of mouse embryos and human embryos may not be identical. Therefore, this data  
275 cannot directly reflect the culture effect on human embryos. Nonetheless, the result still provides valuable  
276 reference for studying human embryonic development and clinical applications. Further validation through  
277 clinical trials is necessary for our device.

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## 5. Conclusion

In this study, we propose a novel system and method to address the shortcomings of the gas system in existing embryo incubators. By investigating the gas mixing and distribution process, theoretical calculation of the inflow volume of supply gases, and determination of mixing parameters, we realized an embryo incubator with the characteristics of high accuracy, fast recovery, and low gas consumption. Additionally, a mouse embryo assay was performed in the incubator to demonstrate the successful development of mouse embryos from zygote to blastocyst stage. Our method offers a superior alternative gas system for multi-chamber incubators, especially for low gas consumption. Future improvements will focus on enhancing the mixing efficiency and preheating the gas before entering the chamber. Given the universality of the method, we anticipate its potential expansion into other applications beyond life science, such as in the industrial and energy sectors.

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## Conflict of interest

The authors declare no conflict of interest.

## Ethical statement

All animal procedures were approved by the Experimental Animal Welfare Ethics Committee of Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences.

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