Long-Term Cell Culture on a Microscope Stage: The Carrel Flask Revisited

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BIOGRAPHY

David Stevenson earned his BSc in biochemistry and molecular biology at the University of Georgia, USA, in 1999, and his PhD in bioengineering at the University of Strathclyde, Glasgow, in 2004. He has been a postdoctoral fellow at the University of St Andrews since 2004. His current research involves laser cell injection, laser-guided neuron growth, and laser cell micropatterning.

ABSTRACT

Engineering the conditions to keep mammalian cells viable on a microscopy stage can be challenging, as a number of environmental conditions must be satisfied in the small amount of space a typical microscopy stage can provide. This technical review suggests a robust solution, based on technology invented in 1912 and used commonly throughout the early to mid part of the 20th century – the Carrel flask. Adapted for modern use, the hermetically sealed glass chamber keeps a CO_2 atmosphere for many days, simplifying one of the most frustrating aspects of stage design and offering numerous other advantages over Petri dishes. The design, manufacture, maintenance, and application of this device are detailed, along with practical advice for long-term on-stage cell culture.

KEYWORDS

light microscopy, confocal laser scanning microscopy, optical trapping, XYZT analysis, 4D analysis, bioreactor, long-term cell culture

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INTRODUCTION

Long term imaging of mammalian cells on a microscopy stage is essential in order to explore a wide range of cellular processes by light microscopy, but it poses a number of bioengineering challenges. Live mammalian cells require a clean, sterile, endotoxin-free environment, with a CO_2 atmosphere (typically 5%) and 37°C temperature. Cells also typically need to be passaged every 48-72 hours in order to prevent cultures from becoming nonviable from over confluency.

In this article, a simple and inexpensive system for the long term microscopic analysis of a mammalian cell culture is described. An on-stage bioreactor, based on a modified design of the Carrel Flask [1,2], provides an inexpensive and robust solution to keeping mammalian cell cultures alive for many weeks and months during microscopy observation.

In particular, the hermetic seal typical of this device simplifies one of the most challenging aspects of long term on-stage microscopy: the maintenance of pH by a physiological atmosphere of CO_2. The biologist will enjoy a device that is compatible with routine sterile cell culture practice; the physicist and engineer will appreciate a device that is compatible with high numerical aperture optics demanded in confocal laser scanning microscopy, optical trapping, and Raman microspectroscopy.

MATERIALS AND METHODS

A glassblower was commissioned to make a modified Carrel flask based on a design common in the early to mid 1900s [1,2] but with two key differences (Figure 1): 1. Instead of being sealed by a rubber bung, the neck was threaded to mate with an autoclavable Schott-Duran GL18 PBT threaded cap with a PTFE seal (Duran, Germany). 2. A 15-mm hole was fashioned in the base. To this hole, a 120-µm thick ('type 0'), 23-mm diameter coverslip was adhered using epoxy resin.

The dimensions of the Modified Carrel Flask were: height: 24 mm (to clear condenser); diameter: 56 mm (to fit most modern stage adaptors); glass thickness: 2.5 mm; length of side arm: 25 mm; outer diameter of side arm: 16 mm; internal diameter of side arm: 11 mm. The angle of the side arm was set to allow access of a 10-mm diameter pipette tip into the centre of the bottom of the device, as some cell-types require this for passaging.

Prior to use, the chamber was cleaned and autoclaved according to the procedure outlined in Table 1. The dry autoclaving procedure in the protocol is of particular importance as it ensures the removal of endotoxins. These small molecules adhere tenaciously to untreated surfaces and are primarily comprised of lipopolysaccharides, ubiquitous cell wall remnants of lysed gram-negative bacte-

Figure 1:
Left: An early Carrel flask design, common to cell culture during the last century. Right: The modified Carrel flask, suitable for high numerical aperture optics. The modified Carrel Flask is 56 mm in diameter.

ria, which are known to decrease the viability of mammalian cells by provoking inflammatory responses [3]. Before cell seeding, the coverslip was coated with 7 µg cm^{-2} of collagen (Sigma, UK). A suspension of cells from the NG108 cell line, a neuron like mouse-rat hybridoma, was seeded into the modified Carrel flask to demonstrate its ability to maintain long term on-stage viability.

A Nikon TE2000 microscope fitted with a 10× 0.3 NA Plan Fluor DIC air objective and a Prior Scientific ProScan II stage was employed for imaging. Images were acquired by LabView (National Instruments). With a 24 mm height, the flask cleared the condenser of both this objective, and higher NA DIC and phase contrast (100×) objectives (Figure 3). In principle, the flask could be designed with a smaller working height when space is constrained.

RESULTS

A modified Carrel Flask was prepared according to the protocol in Table 1. It was then seeded with the NG108 cell-line, left in a temperature controlled 5% CO_2 incubator for 12 hours with a loose cap, sealed under this atmosphere, and placed onto a heated microscopy stage.

Figure 2a-c show cells 1, 24 and 48 hours after placement onto the stage, respectively. Cells displayed good morphology, with no apparent signs of stress such as blebbing or granularity. There was also no obvious change in the colour of the phenol red containing cell medium, indicating that the pH of the culture remained normal. By 48 hours, cells were ready to be passaged (Figure 2c).

Our laboratory has kept this particular cell type going for many weeks in this manner. In fact, the termination of an experiment employing a modified Carrel flask was usually due to the fact that the cells had exceeded their useful passage limit, as opposed to on-stage death or loss of sterility.

DISCUSSION

The many advantages of using a modified Carrel flask over a Petri dish are outlined in Table 2. The modified Carrel flask, after an initial cleaning and seeding phase, may be hermetically sealed, placed onto a heated microscopy stage, and left without concern for CO_2 purging. In contrast, when using a coverslip bottomed Petri dish a 5% CO_2 atmosphere must either constantly be purged on the stage, or the entire stage assembly must be hermetically sealed. The former method is dangerous, and the latter impractical and expensive. Actively purging 5% CO_2 into a laboratory can also lead to 'air hunger' (symptoms of which include breathlessness, nausea, and dizziness) [4] and exceeds occupational exposure limits in many countries. Slightly higher concentrations can lead to unconsciousness and asphyxiation.

Every 48 to 72 hours (depending on the confluency of the cells), the flask may be removed from the microscopy stage, and the cells passaged in a sterile hood, allowing the same cell line to be observed over multiple passages. After passaging, and before returning to the heated microscopy stage, the flask is purged with CO_2 by placing it, with a loose cap, into a standard cell culture incubator. After a suitable time of gas equilibration, the device is removed from the incubator and the cap immediately sealed. Sealing the cells under the same atmosphere they are routinely cultured in prevents the cells from having to adapt to any subtle gas concentration differences that a separate gassing system might be in danger of providing, were the two systems not calibrated to exactly match. The flask may now be transported back to the microscopy stage for further analysis.

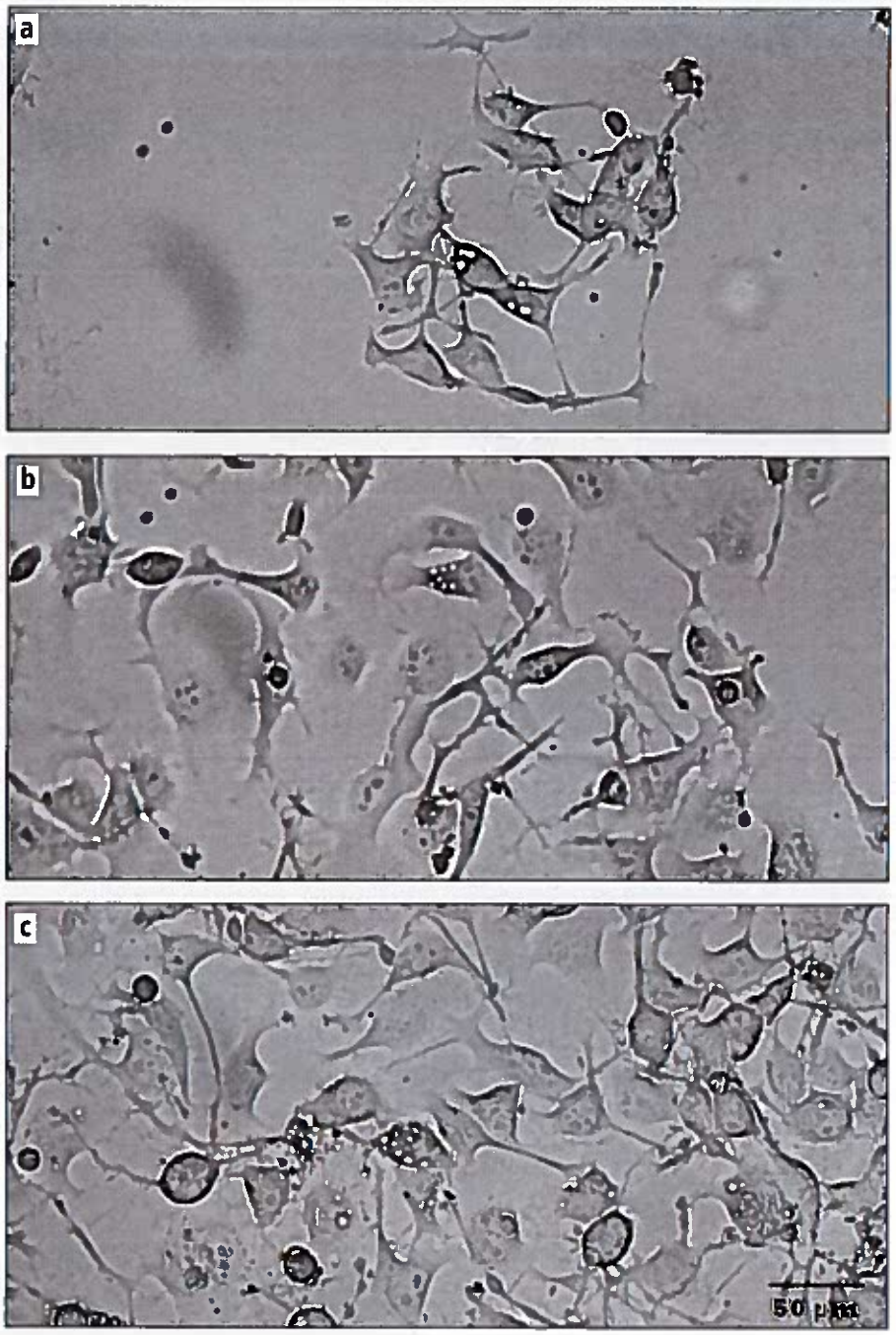

Figure 2:
NG108 mammalian cell line, seeded into a modified Carrel flask, left with a loose cap for 12h in a 5% CO_2 incubator, sealed, and left on a heated microscopy stage for (a) 1 hour, (b) 24 hours, and (c) 48 hours.

Unlike Petri dishes that suffer from wicking of the cell culture medium caused by any knocks during transportation, the modified Carrel flask remains sterile during movement. This can be of particular advantage if the microscopy stage is far away from the cell culture facilities, as is often the case in multi-disciplinary projects. In principle, cultures could be maintained in a 37°C battery-operated portable heater for longer journeys.

Depending on the application, the coverslip may be replaced. For example, different microscope objectives require different coverslip thicknesses, and certain applications such as Raman microspectroscopy require specialist coverslips such as quartz. In our hands, the thinnest coverslip on the market, at a mere 120 µm ('type 0'), survives without breaking over many weeks of cell passaging. One thing to note, however, is that dragging the flask across a surface should be avoided as it can scratch the bottom surface and lead to decreased image quality.

Long term monitoring of CO_2, O_2, pH, temperature, etc., can also be realised with the modified Carrel flask by simply drilling a hole in the autoclavable cap, and sealing the autoclavable probe of interest through the hole with epoxy resin. Cap-based probe assemblies could then be pre-autoclaved, and swapped on the flask arm under sterile conditions depending on the needs of the experiment. For example, a standard 'type T' thermocouple could easily fit through a cap, or a mini-pH probe such as the needle probes marketed by Diamond General Development Corporation. If multiple probes of interest are desired and the probes are too large to be accommodated through a single cap, there is no reason the flask could not be altered to have multiple arms with multiple caps. We predict that our own design could be manufactured with six different side arms if necessary.

Heating the stage can be performed by a number of methods. In our experience, a combination of a perspex heating chamber (Solent Scientific) and an objective heater (Minco Kapton Thermofoil, on an oil immersion objective) works very well to obtain a homogeneous temperature profile (37±0.4°C). If space is limited, the modified Carrel flask fits (by design) onto a commercially available doughnut Peltier (55 mm outer diameter, 27 mm inner diameter, 3.8 mm height) (Melcore, USA; product number RH 1.4-32-06).

CONCLUSIONS

We have presented a system of on-stage cell culture that is robust in its adaptability to the many needs of on-stage long term cell culture. The modified Carrel flask has served our laboratory very well in recent years, and it was the excitement about this device from microscopists we have met who are frustrated with long term imaging experiments that led us to write this article.

Although it is difficult to praise Alexis Carrel without hesitation, as he was an extraordinarily controversial character in history, his contribution to cell biology in the early part of the 20th century is indisputable. He won the Nobel Prize for physiology or medicine in 1912, and he is infamously claimed to have kept fetal chicken heart cells alive in a Carrel Flask for over 30 years. Controversy aside, and almost a century on, we hope we have shown that sometimes a new problem can be solved by adapting a classic solution.

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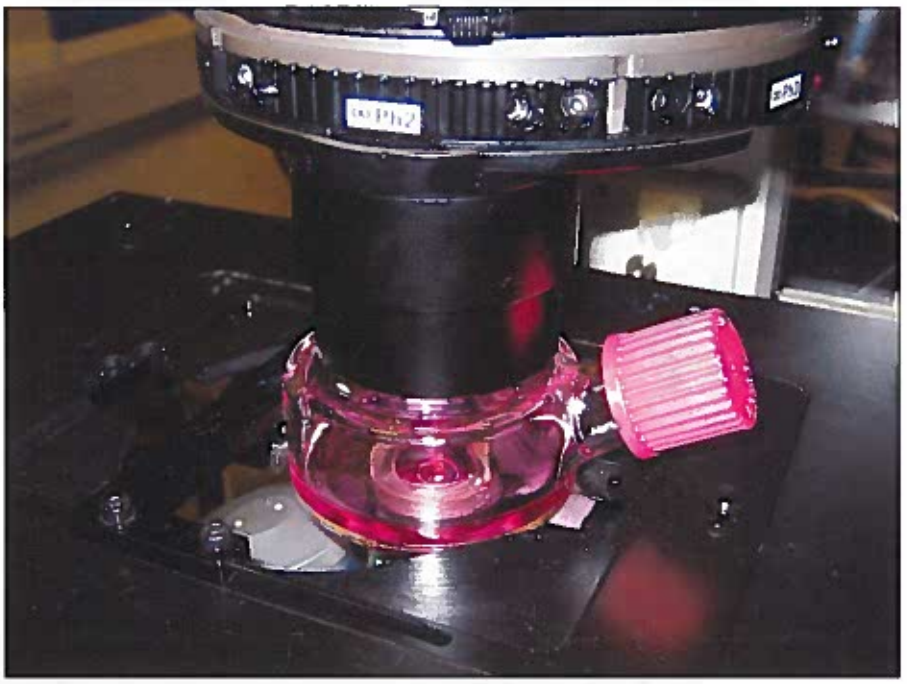

Figure 3:
The modified Carrel flask on the stage of a Nikon TE2000 microscope.

#	PROTOCOL
1	If replacing old coverslip, soak in acetone for 4 h for easy removal (optional)
2	Soak in Decon 90 or equivalent for 24 hours
3	Glue new coverslip onto flask using epoxy resin (optional)
4	Rinse with sterile 18 M-ohm water, then Analar grade acetone, then isopropyl alcohol
5	Dry autoclave at 250 °C for 3 h to remove endotoxins
6	In sterile hood, rinse with sterile 18 mega-ohm water
7	In sterile hood, coat coverslip with desired extracellular matrix protein
8	In sterile hood, rinse with desired cell medium
9	In sterile hood, seed cells
10	Purge flask with desired CO_2 conc., e.g. by placing into a cell incubator with a loose cap
11	Seal the cap tightly and place onto heated microscopy stage
12	When cells need to be passaged, treat modified Carrel flask as normal culture flask
13	When not on heated stage, place modified Carrel flask in incubator with loose cap

Table 1:
Protocol for preparing and using the modified Carrel flask for long term on-stage microscopy.

#	DESIRED FEATURE	MCF	GPD
1	Maintain a homogeneous temperature of 37 °C	Yes	Yes
2	Maintain an atmosphere of 5-10% CO_2	Yes	No
3	Maintain sterility during analysis and cell passaging	Yes	No
4	Maintain isotonicity (by not allowing excessive evaporation)	Yes	No
5	Be free from endotoxins	Yes	Yes
6	Be compatible with high NA optics	Yes	Yes
7	Be compatible with common microscope XYZ stage adaptors	Yes	Yes
8	Be easy to transport without contaminating cell cultures	Yes	No
9	Allow sterile access of CO_2, temperature, and pH probes	Yes	No
10	Have a low height profile to allow for condenser clearance	Yes	Yes
11	Have a low, long-term operating cost	Yes	No

Table 2:
Desirable features of an on-stage bioreactor. MCF = modified Carrel flask; GPD = glass-bottomed Petri dish.