

## Transporting cells over several days without dry-ice

Sally P Wheatley<sup>1,\*</sup> and Denys N Wheatley<sup>2</sup>

<sup>1</sup>School of Life Sciences, University of Nottingham, Queen's Medical Centre, Nottingham  
NG7 2UH, UK

<sup>2</sup>BioMedES, AB51 0LX, UK

\*Author for correspondence

**Keywords:** cell culture, LMT agarose, shipment

**Summary statement:** This paper describes an easy dry ice-free method of sending cultured cells across the globe at ambient temperatures.

## Abstract

This paper describes a simple, hazard-free and inexpensive procedure that allows researchers to send cultured cells across the globe at ambient temperatures. The method enables transit of up to 2 weeks without compromising cell recovery. Its use will assist collaborators in distant laboratories to exchange cells without using dry-ice.

## Introduction

For many decades, the conventional method of sending cells from A to B globally has been as frozen samples dispatched by courier in several kilograms of dry-ice. From storage to delivery, the cost is high and the procedure hazardous with many couriers refusing to take cargo on dry-ice. Delay in transit is common (e.g. slow customs inspection), and when this occurs the dry-ice often evaporates, leaving the cells bathed in cryoprotectant (10% DMSO), which is cytotoxic at ambient temperatures. While cell culture facilities will most likely continue to use this mode of shipping because their collections are held in liquid nitrogen, other methods are becoming available that minimise these frustrations and maximise cell recovery post-transit. They also have the added advantage of eliminating large CO<sub>2</sub> emission associated with using dry-ice. Some researchers simply send culture flasks of cells filled with medium and tightly secured caps, but here agitation can cause cell monolayers to detach; but, more importantly, liquids pose a spill hazard, and are prohibited in the mail and air-travel. To circumvent these issues, we and others have turned to gel-based medium to transport biological samples, as published in a broad patent in 2002 (Baust, Buskirk and Baust, 2002), and found in (Bagasra O, Berman W, Hashemi S, Howeedy A, 1985) (Brendel MD1, Kong SS, Alejandro R, 1994) (Stevens *et al.*, 2005). Undoubtedly there is a need for a simple, inexpensive and non-hazardous method of transporting cell cultures between collaborators, particularly in this era in which designer cell lines are becoming centre-stage. What follows is a brief description of a tried and tested method we have devised that can fulfil this need.

## Materials and Methods

### Cell Culture

All cells (having been tested as mycoplasma-free) were cultured in DMEM with 10% fetal calf serum (FCS), supplemented with 1% glutamine and 1% anti-anti, here-in-after referred to as “1 x complete DMEM”. Cells were maintained at 37°C in a humid incubator with 5% CO<sub>2</sub> in air. Prior to transit, cells were harvested by trypsinization, pelleted by centrifugation, and resuspended at 0.5-1 x 10<sup>7</sup> cells/ml in 1 x complete DMEM. Rat astrocytes were isolated (ethical approval AWERB ref 000148) and cultured in DMEM supplemented with 500 ug/ml proline (James *et al.*, 2011). Human myoblasts were isolated with University of Nottingham ethical approval (G11092014SoLS) and cultured in F10/HAMS with 20% FCS(Leary *et al.*, 2018). Cell culture reagents were supplied by Thermo-Fisher, unless stated otherwise. The following cell lines have been used, although not all will be referred to in this report:

#### Normal

RPE-1: human retinal pigmented epithelial  
3T3: murine fibroblast  
MRC5: human lung fibroblasts  
HEK293: human embryonic kidney

#### Malignant (human)

HeLa: cervical  
U2OS: osteosarcoma  
Hct116: colorectal  
MDA-MB231: breast  
SKBR3: breast  
EJ30: bladder  
MCF7: breast

#### Primary

Rat astrocytes (gift from A Bennett, SoLS, Nottingham)

Human myoblasts (Leary *et al.*, 2018) (gift from K. Tsintzas, SoLS, Nottingham)

#### Preparation of “Transporter”

A 2% solution of low melting temperature (LMT) agarose (Sigma catalogue number A9414) was prepared in PBS, sterilised by autoclaving, cooled and supplemented with filter-sterilised HEPES at 20mM (pH7.4), then held at 37°C ready for the addition of cells. Reagents were supplied by Sigma-Aldrich.

#### Cells in “Transporter”

Cells at 1 x 10<sup>7</sup> cells/ml resuspended in complete DMEM were mixed 1:1 with pre-warmed transporter, pipetted into 1.5 ml microfuge tubes in ~100µl aliquots, and sealed with parafilm (Figure S1C). When using PBS as the diluent for LMT agarose, 1 x

complete medium normally used for the particular cell type is recommended. If LMT is constituted in water, then 2 x complete medium should be used to ensure the carrier is isotonic, however, this will require medium to be prepared from powdered stock.

The tubes were put into “Jiffy” bags (size A) and sent to their destinations by regular mail, air-mail or courier, with associated documentation.

## **Recovery**

Upon receipt, the microfuge tubes containing the cells are placed on a heat-block at 37°C, and 1 ml of pre-warmed complete medium was added, and the gel pellet thoroughly dispersed by pipetting intermittently for 5-10 min. The cells were then seeded on to Petri dishes, well plates, culture flasks or onto live imaging chambers with pre-warmed medium in the usual manner.

## **Resazurin Assay**

To assess cell recovery, cell number was monitored using a standard resazurin assay. Briefly, cells were seeded into 96-well plates in quadruplicate, and incubated for 1h at 37°C with 10 µg/ml resazurin diluted in complete DMEM before being read on a Fluostar Galaxy spectrophotometer with excitation set at 530 nm and emission to 590 nm. Note that each cell line has a different metabolic profile (Figure S1A), therefore heights of the bars should only be compared within the dataset for a single cell type.

## **Imaging**

**Phase:** Cell attachment and morphology was followed using a standard inverted Nikon microscope fitted with a x 20 or x 40 objective, and imaged in phase-contrast using a Nikon D3000 camera.

**Fluorescence imaging:** Primary cells, grown in Ibidi live imaging chambers, were washed with pre-warmed PBS, fixed with 4% formaldehyde/PBS (5 min, 37°C), permeabilised with 0.15% triton/PBS (2 min, 37°C), blocked with PBS/1%BSA/azide (15 min, RT) and immunostained to visualise the astrocyte specific marker glial fibrillary acid protein (GFAP), with anti-GFAP antibodies, (Cell Signalling, mouse monoclonal GA5; 1/300; 1h, room temperature), followed by 1/200 fluorescein conjugated anti-mouse (Vector, 1/200). Images were acquired using an Olympus IX71

microscope fitted with a x 40 (NA 1.3) oil objective, using Deltavision software and a CoolSnap camera. A single deconvolved 0.3  $\mu$ m optical section is shown.

TIFF images were prepared in Image J.

## Results and Discussion

First, to demonstrate the risk of sending cells on dry-ice, 3 cell lines were resuspended in 90% FCS with 10% DMSO and held on dry-ice for 7 days. For one vial of each line, the dry-ice was regularly replenished, but for the other samples it evaporated completely within ~4 days. Cell viability was assessed 72h post-seeding, using the metabolic resazurin-based assay. Recovery from frozen was successful when the dry-ice was maintained throughout transit, but recovery was poor (RPE) or non-existent (U2OS, HeLa) when the dry-ice had expired (Figure 1A).

We have compared the efficacy of recovery between cells held on dry-ice and those in our “Transporter”. Four cell lines were resuspended in either 90% FCS with 10% DMSO and placed on dry-ice (replenished) or in Transporter held at constant temperatures of 0 (ice), 4, ~20 (ambient), or 37°C. After 72h incubation, cell viability was assessed using resazurin. All cell lines recovered better in transporter than on dry-ice when previously kept at 0, 4 or 20°C (Figure 1B), which is also evident from their morphology when reattached and growing in Petri dishes (Figure 1C). Recovery after 72h transit at 37°C in transporter was cell-line dependent, with untransformed NIH 3T3 (mouse fibroblasts) and RPE cells (human retinal epithelial) recovering successfully (Figure 1B). For transformed cell lines (e.g. HeLa, A549), transportation for 7 days up to 27°C was well tolerated, but temperatures of 32°C and above were not. The most robust cells tolerated up to 3 weeks of transport (e.g. U2OS, MRC5) at ambient temperature (20-22°C). A summary of similar findings using 8 different cell lines kept for different lengths of time in Transporter is shown in Table 1. [However, not all cell lines will necessarily respond in the exactly the same way, and thus it is worth checking carefully beforehand their ability to endure the conditions of transportation.]

Further experiments were carried out to determine the optimal cell density and maximum duration tolerated in transit. To determine the optimum cell density, we assessed cell viability post-transit for 3 cell lines transported for 72h at ambient temperature at  $10^6$ ,  $10 \times 10^6$  or  $20 \times 10^6$  cells per ml. Notably, although RPE and U2OS cells fared similarly at all densities, HeLa cells did not survive well at  $20 \times 10^6$  cells/ml (Figure 1D). When transit was carried out at  $4^\circ\text{C}$ , all 3 lines did less well at  $10^6$  cells/ml (data not shown); therefore subsequent experiments were carried out with cells at between  $10^6$ - $10^7$  cells per ml, with the preferred density being  $5 \times 10^6$  cells/ml. Growth curves for HeLa and U2OS cells recovering after 3 days storage in Transporter at different temperatures are shown in Figure 1E and F. When cell viability was compared between cells transported for 72h versus 240h, for all 4 cell lines tested there was a decline in cell viability (Figure S1B), indicating that shorter transit is better and providing some evidence that these cells do not proliferate when transported at ambient temperature.

Having established that immortalised cell lines could tolerate transportation in this way, we next asked whether primary cells would also survive. Rat astrocytes (Figure 2A, B), and human myoblasts (Figure 2B), were seeded 24h prior to imaging either directly from the stock flasks (no transportation) or after 72h in transporter. Phase imaging demonstrated that recovery was excellent in each case and cells looked similar regardless of whether they had been transported or simply passaged. Moreover, to prove that astrocytes retained their normal and defining characteristics after transport, we immunostained them with anti-GFAP antibodies and observed an extensive filamentous network (Figure 2B). Thus transporter can also be used for primary cell transit, at least for up to 72h.

Given that organisms such as zebrafish (Percival and Parant, 2016), organoids and eggs (Udan *et al.*, 2014) are routinely immobilised in agarose for analysis by live fluorescence confocal and light sheet imaging, it is unsurprising that this method is so effective. Indeed, our Transporter medium is similar in many respects to the natural gel of amphibian eggs, which supports development to the juvenile stage of the animal. Our Transporter has successfully supported the carriage of developing *Drosophila* (data not shown). Thus it will be interesting to determine whether this method can aid the transit of germ cells (Hatakeyama, 2017) - which would aid breeding programmes - and stem cells, which would be medically beneficial, as also to others, such as

veterinarians. Should transporter support the preservation of small pieces of tissues (biopsies and organs), this would have huge medical implications, with the most immediate benefactors being transplant patients. And finally, Transporter could also be adopted to improve containment of species presenting biological safety hazards that need to be shipped across the globe for diagnostic purposes.

## **Conclusion**

We have disclosed a simple, inexpensive and non-hazardous method for sending cultured cells between laboratories. While there is some variation in survival depending upon cell type, excellent recovery is seen when cells are transported at  $5 \times 10^6$  cells/ml within the temperature range 4-27°C for 7 days, and where tested there have been cases of very good recovery after 3 weeks at ambient temperature (20-22°C). For the best results we recommend sending cells in small aliquots of Transporter (100µl) in microfuge tubes at  $5 \times 10^6$  cells/ml, ensuring that the gel pellet is dispersed as fully as possible prior to seeding.

Proof of principle was established on 3 occasions, with cells being taken as hand-luggage in transit (for up to 5 days) from the UK to Hong Kong, where excellent, fast recovery was observed after seeding. The method has since been successfully trialled by at least 7 independent colleagues.

## Cell Transporter Protocol

### Sending:

1. Prepare a 2% solution of low melting temperature (LMT) agarose (Sigma A9414) in PBS, dissolve and sterilise by autoclaving, then cool to 37°C.
2. Under sterile conditions add HEPES to a final concentration of 20 mM (pH7.4), mix, aliquot\* and store, or use immediately.
3. Harvest exponentially growing adherent cells by trypsinisation and re-suspend at  $10^7$  cells/ml in the regular complete medium used for that cell line.
4. Pipette 50µl sterile LMT agarose into a pre-warmed 1.5 ml microfuge tube.
5. Pipette 50µl of the cell suspension into the warm LMT solution and mix gently by pipetting (1:1 cells/LMT ratio). Place on rack at room temperature to cool until gel forms (30-60s).
6. Seal tube with parafilm and place in padded envelope with associated documentation.
7. Send via mail, air-mail or courier, aiming for arrival at destination in preferably less than 1 week.

### Receiving:

1. Under sterile conditions, add 1 ml of pre-warmed complete medium to the microfuge tube, pipette to disperse and break the gel pellet.
2. Incubate at 37°C for a further 15-30 min. Re-pipette until the gel has been fully dispersed.
3. Transfer to awaiting tissue culture vessel and culture using normal procedures.

### Additional Notes:

- 2% LMT/HEPES can be stored at 4-22°C for 2 months. *Do not freeze*. LMT can be re-melted by microwaving gently for several seconds or heating to 60°C. Cool to 37°C before use. 1-2 ml aliquots made in 5 ml tubes are most convenient for cell culture.
- By placing solution on a rack in step 4 (sending), the pellet forms at the bottom of the tube. This minimises evaporation, and, if no longer a discrete pellet at the destination, will suggest that a temperature >37°C was exceeded during transit.
- Optimal density may vary with cell type.
- The procedure can be scaled up, but ensure that there is sufficient space for the addition of 10 volumes of medium upon receipt.
- Efficiency of recovery improves with greater dispersion of the gel pellet.



## **Acknowledgements**

We thank the Chouden Research Foundation (Hong Kong) for funding this project, and especially to Dr Lee Tsun Hou for also providing laboratory support in Hong Kong. We thank Dr. Darina Muthna (Charles University, Prague, Czech Republic), Professor Simon Morley (University of Sussex, UK), Dr. Jekaterina Erenpreisa (Latvia) and Dr Reet Kurg (Estonia), for confirming the methodology. We also thank Dr Andrew Renault for help with *Drosophila* larva. We thank Nikki de Vivo and Andrew Wilhelmsen for assistance with primary cultures.

## **Author Contributions Statement**

SPW: experimental design, execution and wrote MS; DNW: secured funding, participated in trials and edited the MS.

## **Conflicts of interest**

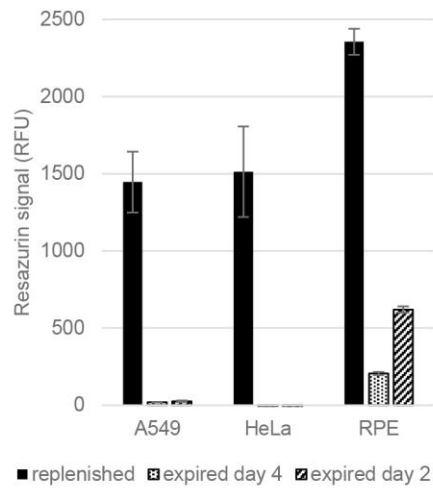
None

## References

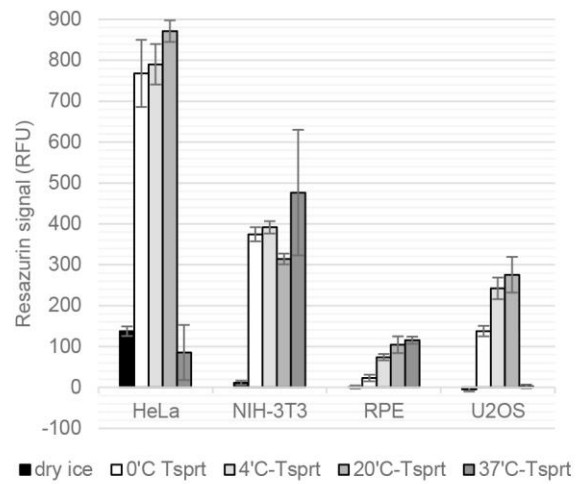
- Bagasra O, Berman W, Hashemi S, and Howeedy AKH. (1985) 'In vitro cloning of tumor stem cells in semi-solid media containing agar and agarose.', *Cancer Immunology Immunotherapy.*, 20(1), pp. 55–60.
- Baust, J., Buskirk, R. Van and Baust, J. (2002) 'The storage of cells, tissues and organs in gel-based media.', *BioLife Solutions Inc. US Patent WO 01/050851*.
- Brendel MD, Kong SS, and Alejandro R, MD. (1994) 'Improved functional survival of human islets of Langerhans in three-dimensional matrix culture.', *Cell Transplant*, 3(5), pp. 427–35.
- Hatakeyama, S. (2017) 'Cryopreservation of very low numbers of spermatozoa from male patients undergoing infertility treatment using agarose capsules', *Human Cell*. Springer Japan, 30(3), pp. 201–208. doi: 10.1007/s13577-017-0166-x.
- James, L R, Andrews, S, Walker, S, De Sousa, P, Ray, A, Russell, NA, Bellamy, TC. (2011) 'High-Throughput Analysis of Calcium Signalling Kinetics in Astrocytes Stimulated with Different Neurotransmitters', 6(10). doi: 10.1371/journal.pone.0026889.
- Leary, MFO, Wallace, GR, Davis, ET, Murphy, DP, Nicholson, T, Bennett, AJ, Tsintzas, K. (2018) 'Obese subcutaneous adipose tissue impairs human myogenesis , particularly in old skeletal muscle , via resistin-mediated activation of NF  $\kappa$  B', pp. 1–13. doi: 10.1038/s41598-018-33840-x.
- Percival, S. and Parant, J. (2016) 'Observing mitotic division and dynamics in a live zebrafish embryo', *J Vis Exp*.
- Stevens, MM, Mayer, M, Anderson, DG, Weibel, DB, Whitesides, GM, Langer, R. (2005) 'Direct patterning of mammalian cells onto porous tissue engineering substrates using agarose stamps', 26, pp. 7636–7641. doi: 10.1016/j.biomaterials.2005.05.001.
- Udan, R S, Piazza, VG, Hsu, C, Hadjantonakis, A, Dickinson, M. E (2014) 'Quantitative imaging of cell dynamics in mouse embryos using light-sheet microscopy', pp. 4406–4414. doi: 10.1242/dev.111021.

## Figures

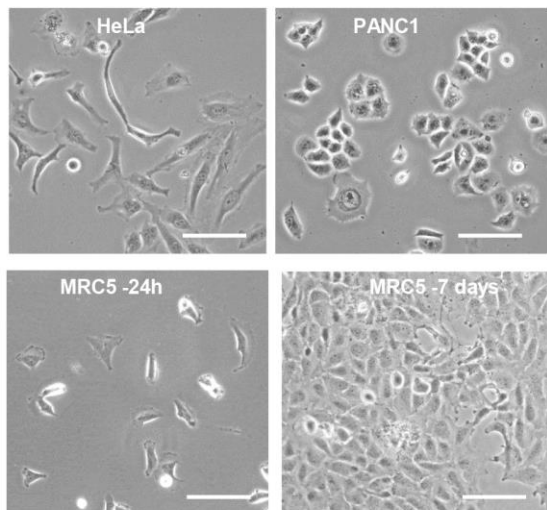
**A**



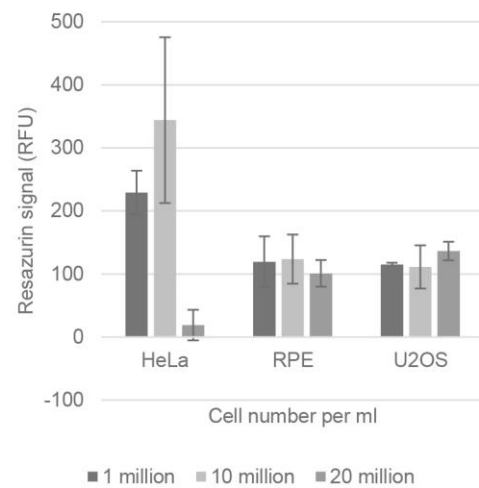
**B**



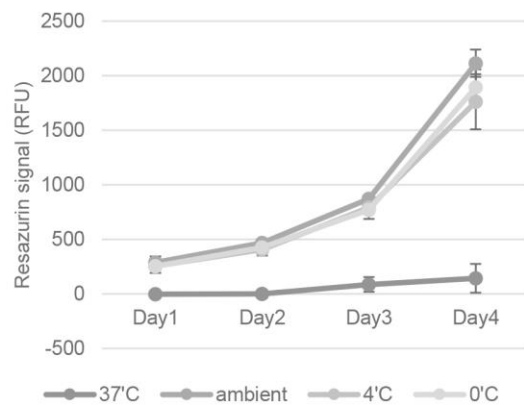
**C**



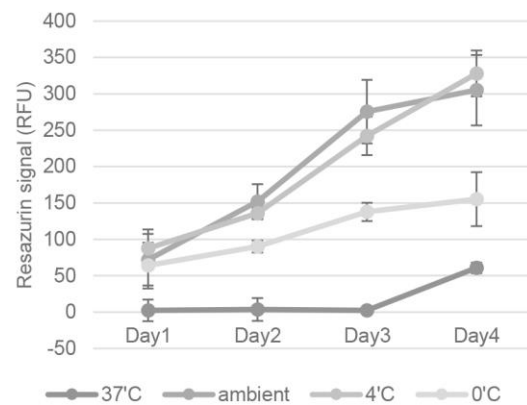
**D**



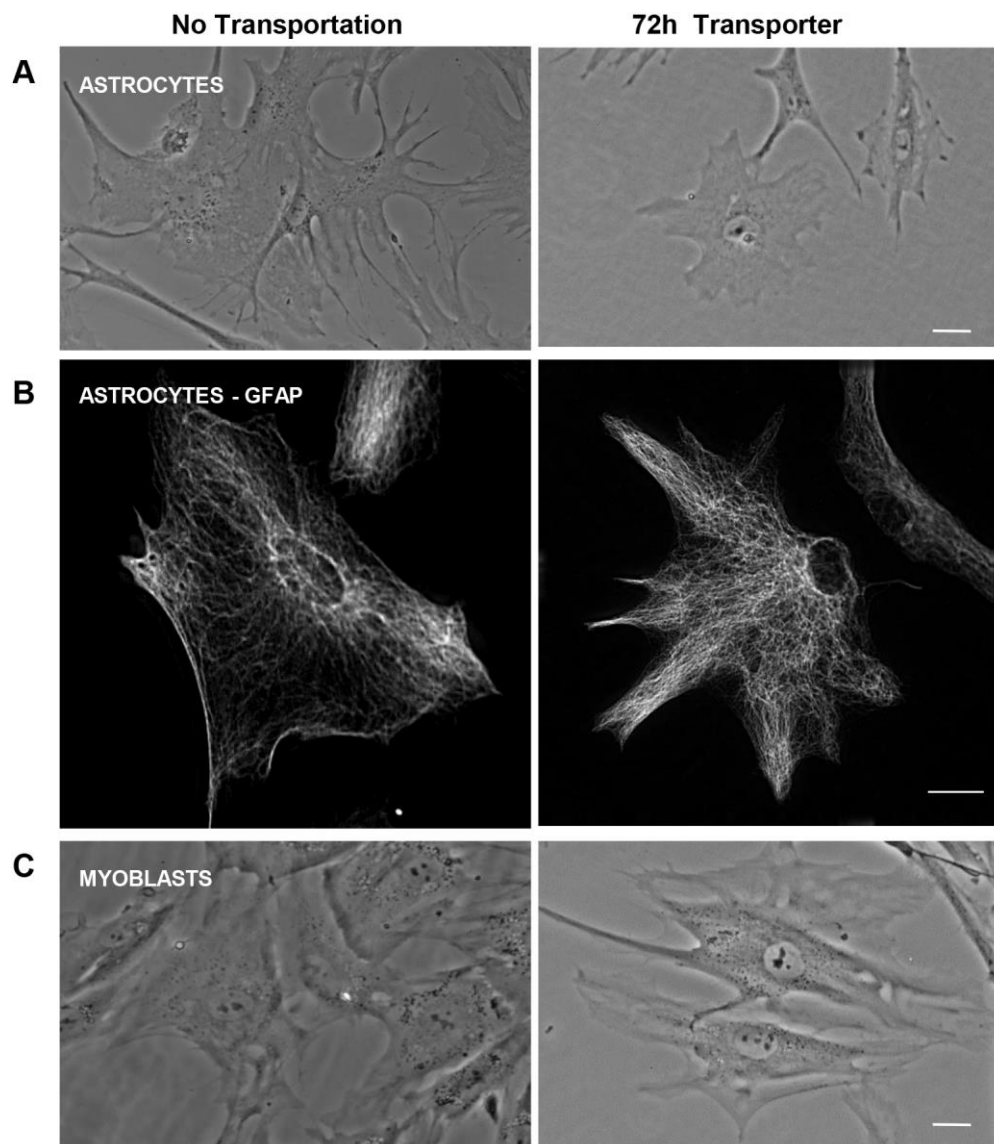
**E**



**F**



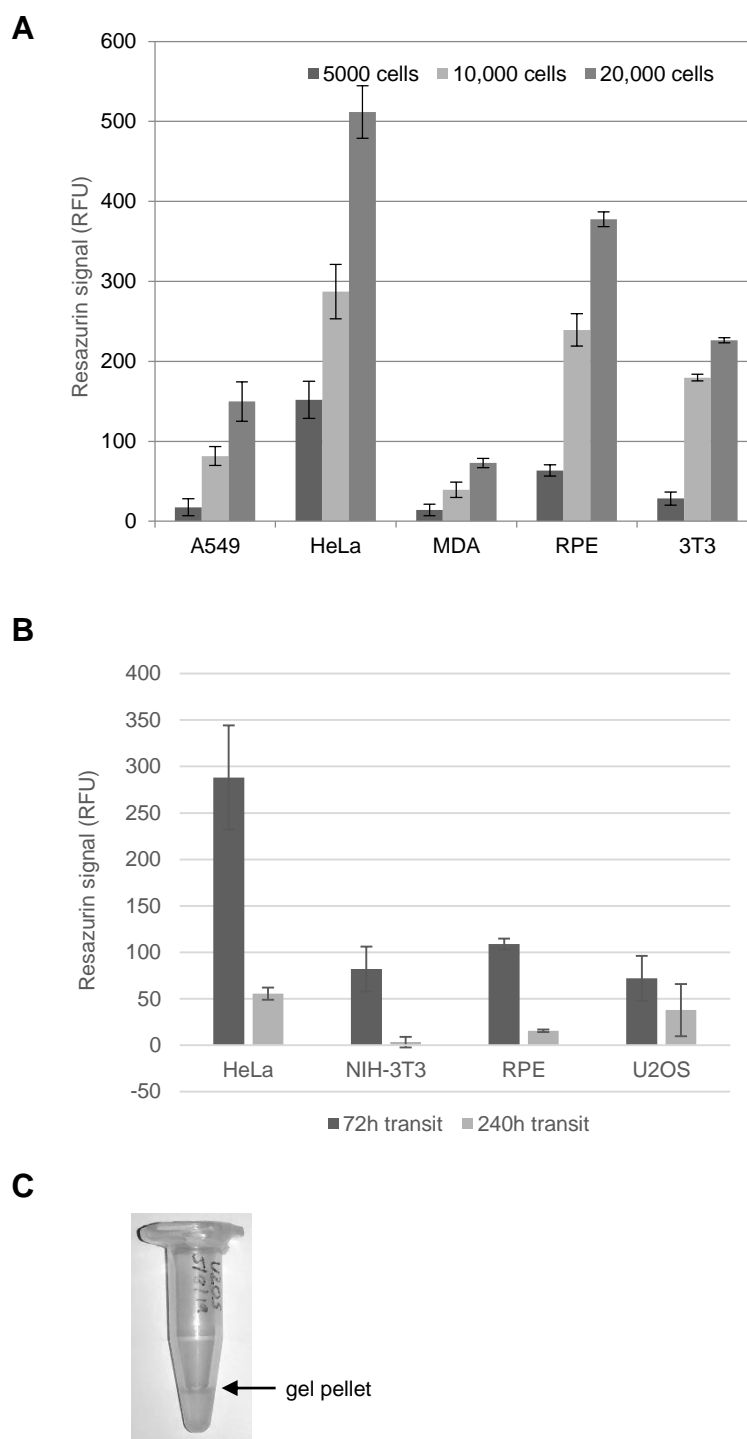
**Figure 1. Cells can be transported without dry ice.** **(A)** Cell lines indicated were incubated on dry-ice in 90% FCS with 10% DMSO for 7 days and cell viability (expressed in relative fluorescence units, RFU) was assessed using the resazurin assay. Scale bars: 100  $\mu$ m. **(B)** The cell lines ( $5 \times 10^6$ /ml) indicated were incubated in FCS/DMSO and held on dry-ice or resuspended in transporter (Tsprt) and incubated at different temperatures before viability was assessed, as in (A). **(C)** phase-contrast images of recovering cells after 7 days in transporter and seeded 72h before imaging (HeLa and PANC2), or 96h in transporter and imaged 24h post-seeding (MRC5). **(D)** Cells were transported at 3 different densities at ambient temperature and cell viability assessed 24h post-seeding. Graphs show mean and SD within a single experiment representative of N=3 independent experiments. **(E & F)** Growth of HeLa **(E)** and U2OS **(F)** cells over 4 days after being kept in Transporter for 3 days at the indicated temperatures. All graphs show representative experiments performed at least 3 independent times. Internal repeats were carried out in quadruplicate, bars/points indicated the mean and error bars show standard deviation within the representative experiment.



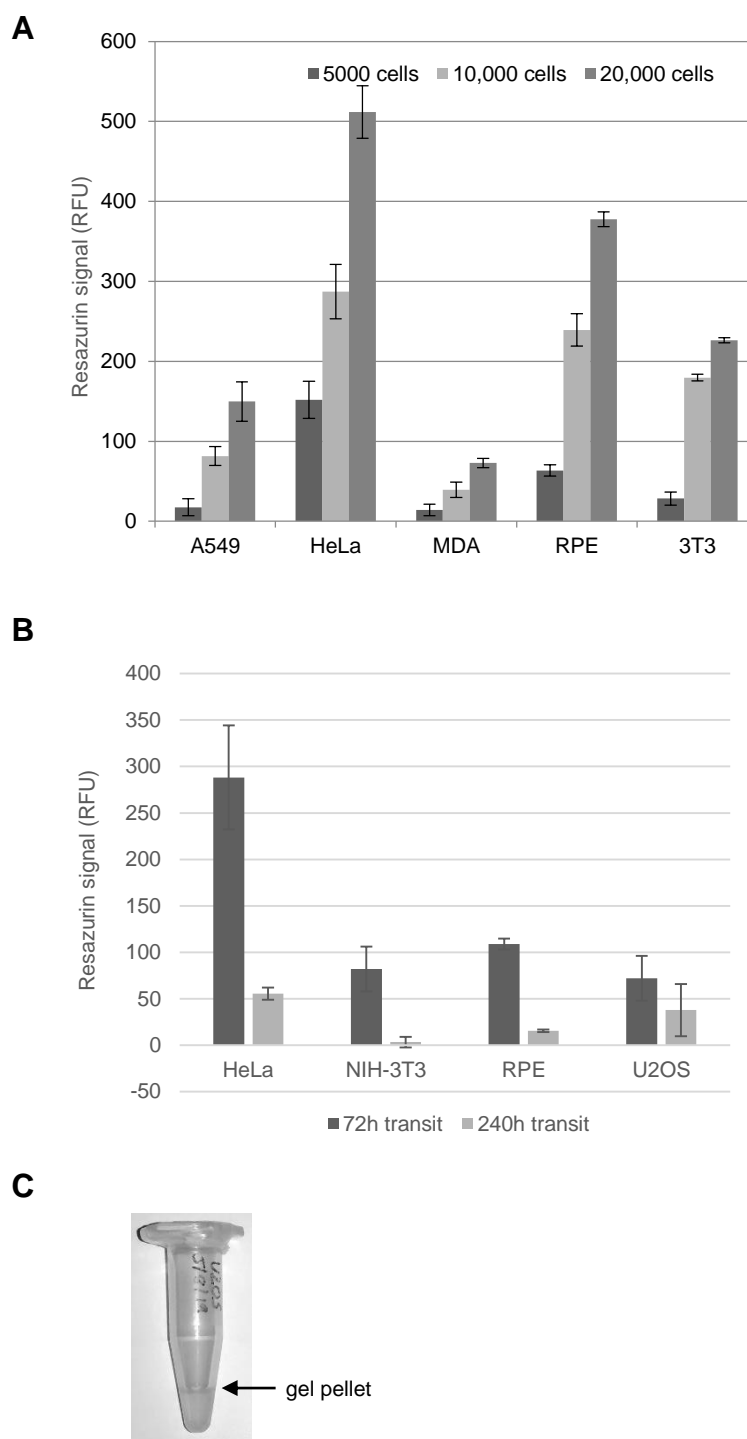
**Figure 2. Primary cells can tolerate 72h transit in transporter.**(A and B) Rat astrocytes were either trypsinised and seeded (no transportation) or incubated in transporter for 72h then seeded, and imaged in phase (A) 24h later. (B) To prove that they retained their astrocytic characteristics, they were fixed and immunostained for GFAP and imaged using a fluorescence microscope. (C) Human myoblasts were treated in the same way as rat astrocytes in (A). Bars 20µm.

**Table 1: Duration, temperature and recovery outcomes of 10 cell types**

Cell line	Type	Duration (days)	Temperature (°C) and recovery
Astrocytes	Rat primary	3	20-22 (yes)
A549	Lung cancer	5	0-4 (yes), 20-22 (yes), 37 (yes)
EJ30	Bladder cancer	5	0-4 (yes), 20-22 (yes), 27 (yes), 32 (poor)
HeLa	Cervical cancer	3-7	0-4 (yes), 20-22 (yes), 37 (poor)
MDA231	Breast cancer	3	0-4 (yes), 20-22 (yes), 27 (yes), 32 (poor)
MRC5	Normal lung fibroblast	5-7	0-4 (yes), 20-22 (yes), 37 (poor)
Myoblasts	Human primary	3	20-22 (yes)
NIH-3T3	Murine fibroblast	3-7	0-4 (yes), 20-22 (yes), 37 (yes)
RPE	Normal human retinal pigmental epithelial	3-7	0-4 (yes), 20-22 (yes), 27 (yes), 32 (poor), 37 (poor)
U2OS	Osteosarcoma	3-5	0-4 (yes), 20-22 (yes), 32 (poor), 37 (no)



**Figure S1. Cell lines have different metabolic profiles and do not proliferate in transit. (A)** 5, 10 or 20 x 10<sup>3</sup> cells were seeded in a 96 well plate in triplicate, allowed to adhere for 4h, then a resazurin assay carried out. **(B)** Cells were seeded after 72h or 240h in transit, allowed 24h to recover then viability assessed. Cell number diminished with increased transit time suggesting loss of viability and no proliferation. **(C)** Microfuge tube containing 100  $\mu$ l cells in Transporter ready for transit.



**Figure S1. Cell lines have different metabolic profiles and do not proliferate in transit. (A)** 5, 10 or 20 x 10<sup>3</sup> cells were seeded in a 96 well plate in triplicate, allowed to adhere for 4h, then a resazurin assay carried out. **(B)** Cells were seeded after 72h or 240h in transit, allowed 24h to recover then viability assessed. Cell number diminished with increased transit time suggesting loss of viability and no proliferation. **(C)** Microfuge tube containing 100  $\mu$ l cells in Transporter ready for transit.