

# Embedding Arabidopsis Plant Cell Suspensions in Low-Melting Agarose Facilitates Altered Gravity Studies

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**Abstract** Gravity plays a role in modulating plant growth and development and its alteration induces changes in these processes. Microgravity research has recently been extended to the use of *in vitro* plant cell cultures which are considered as an ideal model system to study cell proliferation and growth. In general, among the ground-based facilities available for microgravity simulation, the 2D pipette clinostat had been previously considered a suitable facility to be used for unicellular biological models although studies using single plant cell cultures raised some concerns. The incompatibility comes from the standard requirement of shaking a suspension culture for assuring its viability and

active proliferation status in the control samples. Moreover, a related issue applies to the use of the random positioning machine (RPM) for cell suspension experiments. Here, we demonstrate an alternative culture method based on the immobilization of the culture before the altered gravity treatment occurs, such that it behaves as a solid object. Our immobilization procedure preserved plant cell culture viability without compromising basic cell properties as viability, morphology, cell cycle phases distribution, or chromatin organization, when compared with a standard cell suspension under shaking as a control. This approach should allow the space biology community to improve the quantity and quality of plant cell results in future simulated microgravity experiments or spaceflight opportunities.

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## Introduction

Research in microgravity conditions is indispensable to disclose the effects of gravity alterations on any organism. Ideally, this research should be conducted in real microgravity conditions during a spaceflight mission (Des Marais et al. 2008). However, research in the near Earth orbit and access to International Space Station (ISS) is severely constrained by the limited number of flight opportunities and high costs. To overcome these constraints, ground based facilities (GBFs) for simulated microgravity are valuable tools for preparing spaceflight experiments and also for facilitating stand-alone studies (Herranz et al. 2013).

If we want to investigate the effects of altered gravity on plant cell proliferation and growth, a suitable biological

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model system is provided by cell cultures in suspension (Menges, Murray 2002). The two-dimensional (2D) clinostat is a classical method used to simulate microgravity and the use of this method in a special device capable of holding liquids, such as the 2D pipette clinostat, was defined as the system of choice for microgravity simulation studies using unicellular biological model systems (Herranz et al. 2013). It is indeed widely used in cellular and molecular biology experiments (Hemmersbach et al. 2006). However, we have recently demonstrated that the 2D pipette clinostat actually compromises the use of plant cell cultures in simulated microgravity experiments already in the first 24 hours of exposure (Kamal et al. 2015).

An alternative device, the Random Positioning Machine (RPM) is being widely and increasingly used to simulate microgravity in a wide range of experiments involving different biological models (Borst, van Loon 2009; Pardo et al. 2005; Yuge et al. 2003). For the application of the simulated microgravity treatment it is more straightforward when the cells are attached to a surface. Therefore, if cells in suspension are grown on the RPM, they will be subjected to mechanical and shear forces (Leguy et al. 2011). Furthermore, in case of using liquids in the RPM, it is mandatory to avoid gas bubbles, which would result in unwanted fluid motion and associated shear stress to the sample, thus leading to the full invalidation of the simulation experience (Borst, van Loon 2009).

Considering that plant cell suspensions are an essential system in the studies on the effect of microgravity on cell proliferation and growth, we present here a successful method to immobilize an *Arabidopsis* plant cell suspension culture to be used as a solid object in RPM experiments. Our final goals are to check the benefits of using immobilized cells as a proper experimental and control condition in ground based facilities as a basis for upcoming experiments under real microgravity conditions thus exploiting the high potentiality of this biological system with the newest cell and molecular biology methods.

## Material and Methods

### Cultivation of Fast Growing *Arabidopsis* Cell Suspension Cultures (MM2d): Standard Control

*Arabidopsis thaliana* (L.) (Ecotype *Landsberg erecta*) cell suspension culture (MM2d) was grown in Murashige and Skoog medium supplemented with 3 % w/v sucrose, 0.5 mg/L NAA, 0.05mg/L kinetin, with pH adjusted to 5.8 using 1N NaOH (MSS medium). MM2d cells were maintained by weekly sub-culturing (1:20 dilution) every 7 days in 250 mL Pyrex flasks, with continuous shaking at 120rpm in an incubator shaker at 27 °C in darkness (Menges, Murray 2006).

### Immobilization of Cell Suspension Cultures by Embedding in Low Melting Agarose

MM2d cultures were sub-cultured (in a dilution 1:20) at the 7<sup>th</sup> day of growth in fresh MSS medium and kept in a sterile 50 ml Falcon tube in darkness. Low melting agarose (2 % (w/v); gelling at 26–30 °C; SeaPlaque™ Agarose, BDH, Pool, UK) was dissolved in MSS medium in a sterile glass flask by boiling for 10 seconds in a microwave oven and was allowed to cool down to 28–27 °C. The agarose solution was then mixed with an equal volume of the prepared cell suspension, resulting in a final concentration of 1 % (w/v) agarose and a 1:40 dilution of cells in MSS medium. After gentle mixing, 10 ml of agarose-cell mixture were poured into Petri dishes. After the agarose had solidified, Petri dishes were sealed with Micropore™ tape. All steps were carried out at room temperature and under sterile conditions. Then, Petri dishes were kept at 27 °C in dark conditions throughout all steps carried out according to the experimental design (Fig. 1A). At the end of each experiment cells embedded in agarose were recovered and further processed for different types of analyses.

### Cell Viability Test on Fresh Samples

Trypan Blue staining was used to discriminate between live (unstained) and dead cells (Blue stained). A sample of embedded cells was diffused by Trypan Blue dye of an acid azo exclusion medium (Trypan Blue, LONZA) by preparing a 1ml of 0.4 % Trypan Blue solution. Cells were counted under the optical microscope to determine the cell viability rate (stained-living cells/total cells). Cells remained embedded in agarose during the analysis.

### Fixation and Confocal Microscopy

Cells embedded in agarose were fixed by adding 1 ml of 4 % (w/v) paraformaldehyde (PFA, Electron Microscopy Sciences, USA) or 3 % (w/v) glutaraldehyde (Glu, TAAB, England) in phosphate-buffered saline (PBS buffer) onto the surface of the plate for 1 hour. Fixative is able of reaching cells by free-diffusion through the agarose (Ottensmeyer, Huang 1996). After incubation in the fixative, samples were washed with 1 ml PBS buffer for 15 min to prevent over fixation and cytoplasmic extraction (Srinivasan et al. 2002; Start et al. 1992). The agarose including the fixed cells was transferred to 15 ml Falcon tubes and dissolved by immersion in a water bath at 63 °C. Centrifugation of the dissolved solution at 2500 rpm for 5 min allowed the recovery of a pellet of fixed cells without agarose in order to use them for different analytical procedures involving various microscopies (optical, confocal, electron).

For confocal fluorescence microscopy, cells were recovered after PFA fixation. Cell wall was digested using 1 ml of an enzyme cocktail (2 % *w/v* cellulase, 1 % *w/v* pectinase, 0.05 % *w/v* macerozyme, 0.4 % *w/v* mannitol, 1 % *v/v* glycerol and 0.2 % *v/v* Triton X-100, 30 min at 37 °C). Samples were counterstained with 5  $\mu\text{g}/\mu\text{l}$  DAPI (4,6-diamino-2-phenyl-indol) in PBS, for 5 min. After washing with PBS (2 $\times$ 5 min) and with H<sub>2</sub>O (2 $\times$ 5 min), samples were mounted with DABCO and observed under the confocal microscope (Leica TCS SP5 confocal laser scanning microscope). Images were analyzed using the “Leica AF” image analysis software v2.4.

### Sample Freezing and Flow Cytometry

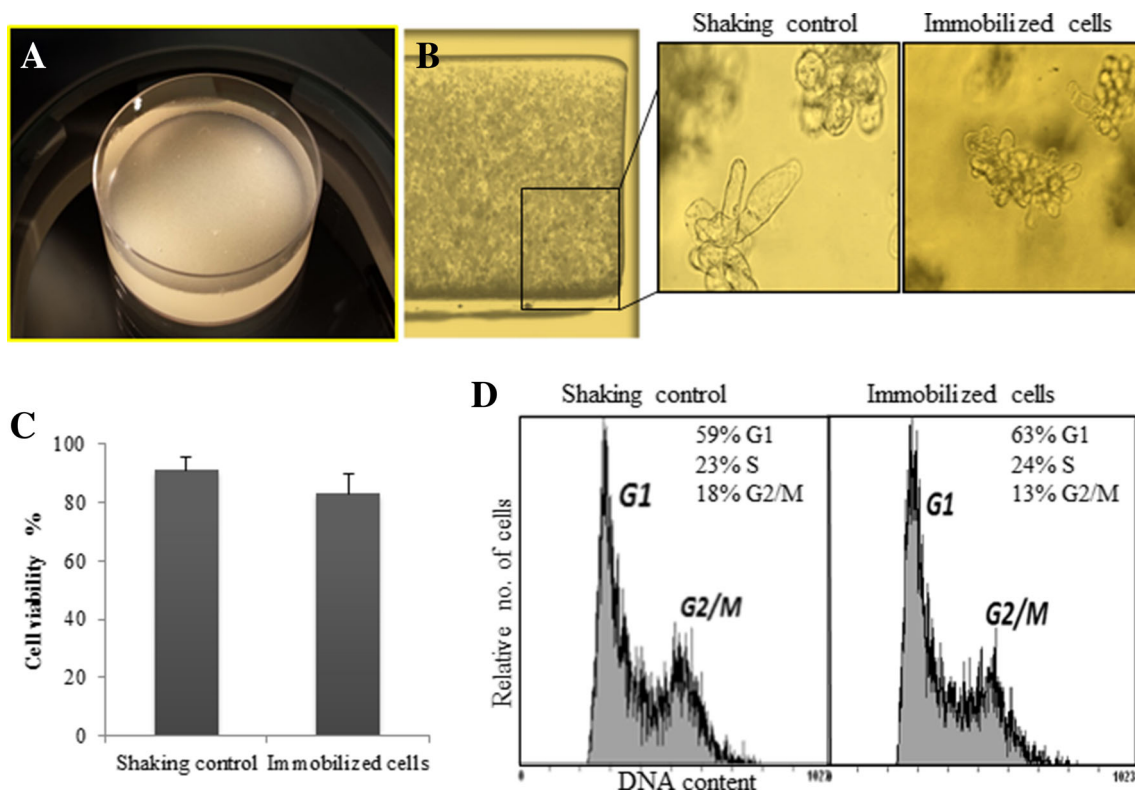
Cells were fixed with 1 ml 1 % (*w/v*) PFA for 15 min, agarose was dissolved at 63 °C using a water bath and cells were extracted by centrifugation, as described above. Then, cells were directly frozen by immersion in liquid nitrogen.

For flow cytometry, each sample of the frozen cell pellet (500 mg) was treated with the High Resolution Kit for plant ploidy level analysis (Kit Cystain UV precise P; Partec

GmbH, Munster, Germany) containing solution A (nuclei extraction buffer) and solution B (staining buffer containing DAPI,) to determine the DNA content. To release cell nuclei, cells were first rinsed in solution A and carefully chopped with a sharp razor blade. Then, cells were filtered and rinsed in solution B (Menges, Murray 2006). On average, 10000 particles in three replicates were counted by flow cytometry (cell sorter FACS Vantage, Becton–Dickinson, San Diego, California), using ion laser tuned at 360 nm and detection of emission using a blue fluorescence emission filter (band pass filter of 424/44 nm). FACS analysis results were analyzed using BD CellQuest™ software to determine the ratios of cell cycle phases according to the DNA content of individual cells (2*n* for phase G1, 2<*n*<4 for S-phase, 4*n* for G2/M phases).

### Statistical Analyses

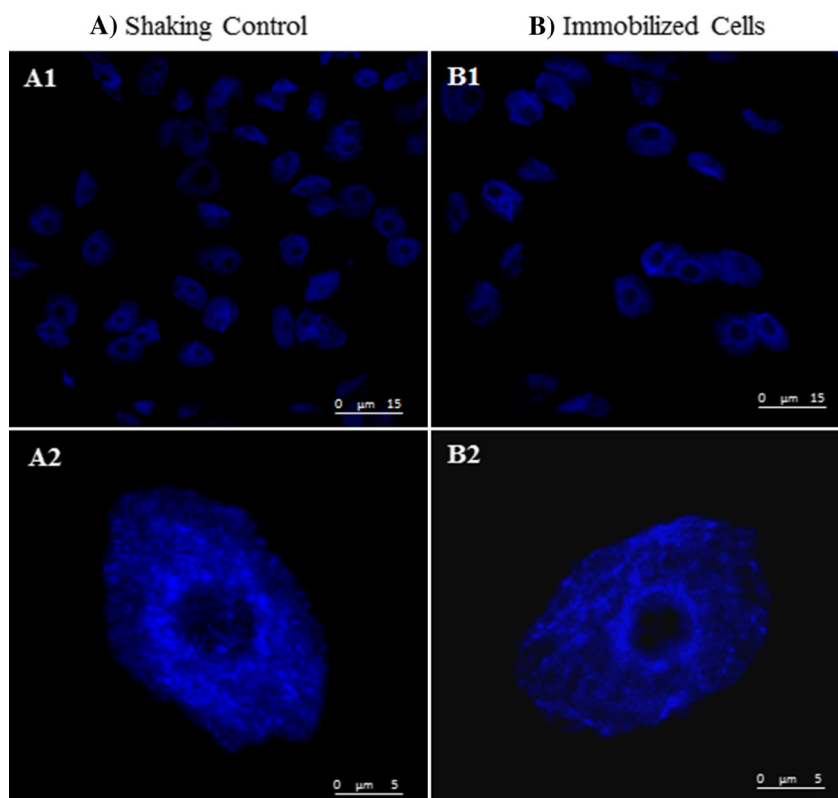
Data were statistically analyzed using SPSS v.22 software. The variance of differences was measured using ANOVA test. Degree of freedom was followed as  $p \leq 0.05$  considered statistically significant (labeled with \*) (Steel 1980).



**Fig. 1** *Arabidopsis* cell suspension embedding in low melting agarose. A) Experimental plate with *Arabidopsis* cells embedded in agarose for 24 hours. B) Lateral view of the distribution of cells and morphological status of the cells embedded in agarose after 24 hours (right) compared with a sample of a standard cell suspension (left). C) Cell viability test using Trypan blue dye. Cell embedding does not

produce any significant alteration in the viability of cells, compared with the standard control. D) Cell cycle analysis by flow cytometry. The distribution of cell cycle phases is not disturbed by the embedding procedure *versus* the standard shaking control. Standard error was calculated using degree of freedom as  $p \leq 0.05$

**Fig. 2** Chromatin mass organization shown by fluorescence after DAPI staining. Fixed cells were recovered after 24 hours. A1) Standard shaking control cells (magnified in A2). B1) Immobilized cultures in agarose (magnified in B2)



## Results

We have developed a procedure capable of using *in vitro* plant cell cultures, originally grown as cell suspensions, such that they behave as solid objects. Once the procedure of immobilization was established, the key point was to define whether or not it had an impact on the viability and functionality of the cells due to mechanical stress or containment on the cells. Furthermore, the ability of the procedure to produce samples differently processed, to be subjected to various biological analysis protocols, was also tested.

First, cells embedded in agarose were tested morphologically using optical microscope. Results revealed that embedding did not affect morphological features, either the cell shape or the cell size, compared with the reference control (shaking standard control in suspension, Fig. 1B). Subsequently, to determine the number of viable cells present in the embedded cells in agarose for 24 hours, we used the Trypan blue dye exclusion test for cell viability, resulting that immobilization did not produce significant differences in this parameter compared with the standard control after 24 hours (Fig. 1C).

The most important feature of *in vitro* suspension cell cultures is the undifferentiated state of the cells and their high proliferative rate. Therefore, they are an ideal system to study cell cycle progression and regulation and the alteration of cell cycle parameters by different factors.

Consequently, we have tested the impact of embedding cells in agarose on the cell proliferation rate and on the proportion of cells in G1, S, and G2/M phases of the cell cycle. This was determined by flow cytometry, i.e. by determination of the DNA content for each individual cell, using frozen samples (Fig. 1D). Data revealed that the distribution of the cell cycle phases after 24 hours of immobilization in agarose was not significantly altered, compared with the standard shaking control conditions.

Finally, the structure of the cell nucleus in immobilized cells was tested by confocal fluorescence analysis using DNA staining with DAPI. In cells embedded in agarose the organization of the chromatin masses, assessed by DAPI staining, was similar to the standard shaking control (Fig. 2).

## Discussion

The need of using stirring and shaking to maintain the suspension of cells was a serious constraint which prevented the use of this highly valuable biological system in experiments of simulated microgravity using ground-based facilities, and even the implementation of this system in future experiments on real microgravity on board of the International Space Station (ISS). Our procedure for the immobilization of the culture will enable the use of plant cell cultures in altered gravity facilities that require specimens

to behave as a solid object. The procedure developed in our laboratory has involved embedding cells in low-temperature gelling agarose to protect the flooding of cells in the absence of the shearing forces. This procedure is a step forward from a method described previously in tobacco BY-2 cells (Sieberer et al. 2007; Sieberer 2009). In that protocol cells could survive, but the authors did not achieve a full isolation of the cells for enabling their further analyses by different methods; instead, they just used agarose layers containing cells for microscopical observations after a fixation procedure. Here, we have indeed achieved a successful extraction of the cells, including removal of agarose. These extracted cells can potentially be used in a wide range of protocols for biological analysis. Samples recovered either fixed or frozen have been tested; flow cytometry technique, considered as the most suitable approach to study the cell cycle, was successfully used with frozen embedded cells, but these samples can be also subjected e.g. to proteomic, or genomic, or transcriptomic techniques. Otherwise, fixed embedded cells were subjected to fluorescence analysis by confocal microscope, as an example of microscopy techniques that can be used with this kind of samples.

Furthermore, the immobilization procedure has proved to preserve plant cell culture viability. Different tests were performed to check the viability of cells after 24 hours from the immobilization. We demonstrated that embedding cells in agarose does not alter the cell viability compared with the standard shaking control, and that the distribution of cell cycle phases was not altered. The same level of preservation was detected with regards to the chromatin organization, evaluated by confocal microscopy analysis.

The use of plant cell cultures is a fundamental system for cellular studies on the effects of microgravity and other environmental factors to be carried out in future spaceflight missions. Immobilizing a plant cell suspension culture, enabling it to be treated as a solid object, is a successful method to spread the use of plant cellular systems in space plant biology. We expect that this methodology will allow our research field to advance quickly by introducing the application of the modern procedures of molecular and cell biology analysis.

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