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Cell electrospinning an *in vitro* and *in vivo* study**

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Keywords: cell electrospinning, in vitro and in vivo studies, in vivo imaging and analysis, mouse models, tissue engineering and regenerative biology/medicine

Scaffolds play a critical role in the engineering of functional tissues and organs; they provide the cells with a structure for forming the required living microenvironment. There are many apporaches for generating functional scaffolds. We recently pioneered the ability to directly electrospin living cells with a polymer containing many of the molecules found in native tissues. Those studies demonstrated that electrospinning cells did not cause any damage and subsequent investigations showed that cells maintain all their functions *in vitro* post-treatment. In this communication we provide further evidence of the safety of this unique platform technology by demonstrating the *in vivo* viability of this direct cell scaffolding technology in a mouse model. These studies, together with our previous work, highlight cell electrospinning as a novel platform biotechnology for directly engineering functional pre-organised three-dimensional tissues, as yet unrivalled by any other direct cell scaffolding approach.

Fibrous architectures such as scaffolds have been explored in the area of tissue engineering and regenerative medicine for many years. [1] Although numerous investigations have demonstrated a wide range of scaffolds having utility in this endeavor, the use of scaffolds as substrates for cell seeding, for the creation of three-dimensional tissues/organs has been limited. These primary limitations stem from the inability to manually seed uniform quantities of cells over a given scaffold architecture, as well as poor and time dependent cellular infiltration into the scaffold (or throughout its entire depth), which ultimately restricts the possible movement of cells across the scaffold in three-dimensions. [2] Additionally these scaffold substrates present the seeded cells in two-dimension to those nutrients in three-dimensions. These limitations have been partially overcome with the addition of another processing step into the living scaffold generation process, namely exploring ultrasonic agitation to the alternative stacking of cell sheets and scaffolds respectively, [3, 4] Although

such work has been successful using nutrient rich *in vitro* environments, this has not been demonstrated *in vivo* where nutrient availability is more variable.

During embryo development, cell division and patterning in three dimensions leads to tissue and organ generation, with a continual process of remodeling occurring as development proceeds. [5] Hence our intention through our discovery of cell electrospinning was to capture native processes as closely as possible by combining cells with the biopolymer and the required molecules to provide all the constituents contained in native tissue, directly forming a living scaffold. Our previous studies using *in vitro* cell assays demonstrated that electrospinning cells did not produce any negative effects, which might prevent the cell functioning as expected when compared to untreated controls. In the work presented in this communication, previous *in vitro* work has been extended with *in vivo* experiments in the mouse followed over a time frame of nearly two weeks. This combination of *in vitro* and *in vivo* validation illustrates the significant promise this platform has in many areas of research and development, from basic biology, biotechnology and pharmaceuticals to the clinic.

Cell electrospinning (CE) [6, 7] works on the principle of applying a high voltage through a DC source to a conducting coaxial needle system placed above a grounded collection grill or rotating mandrel. The coaxial needle system accommodates the flow of a cell suspension in the inner needle, while a low conducting high viscosity biopolymer flows through the outer needle (Figure 1A). The primary reason for exploring a coaxial needle system is based around its ability to utilize the properties of the biopolymer to shield the highly conducting cell suspension from the external electric field, which arises as a result of the electrodes. In addition this biopolymer also forms the matrix required for the cells. The properties of the biopolymer produce stability in the cell electrospinning process, which results in the

production of a continuous cell-bearing fiber, which over time forms a living scaffold or membrane. However cell electrospinning could be carried out in the single needle system in stable conditions by submerging the single needle in a cell-friendly biopolymer, which would provide a controlled atmosphere by which cell spinning would be achieved. [8]

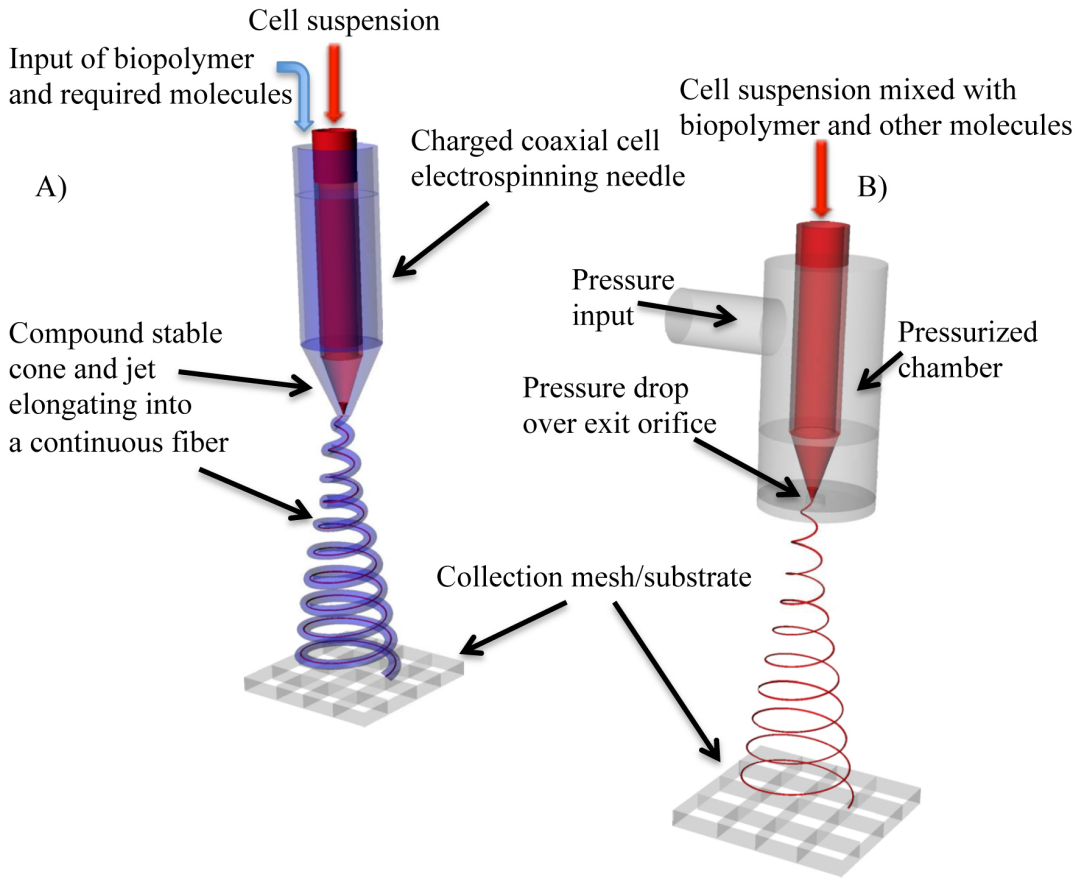


Figure 1. Schematic depiction of the two direct cell spinning approaches discussed in this article, A) cell electrospinning and B) aerodynamically assisted bio-threading.

In these studies we also wished to explore another technology pioneered in 2007 [9, 10], namely aerodynamically assisted bio-threading (AABT – Figure 1B). This technology, which is not electric field-driven exploits a pressure drop over an exit orifice to produce a continuous polymer as a fiber, collection over time forms a scaffold and membrane. The novel advantage

of AABT over CE is that as a pressure drop drives the former, electrical conductivity of the cell suspension does not play a critical role, meaning a single needle could be used, with the cells mixed with a biopolymer (Figure 1B). Although their driving mechanisms are different their capabilities are very similar (Figure 2). One exception may arise when fiber alignment is required; cell electrospinning achieves this with the exploration of the electric field coupled with a given geometry for the grounded electrode. In the case of AABT a rotating mandrel could be used, as in CE, but physical movement of the mandrel would be required for cross stitching fibres in different directions.

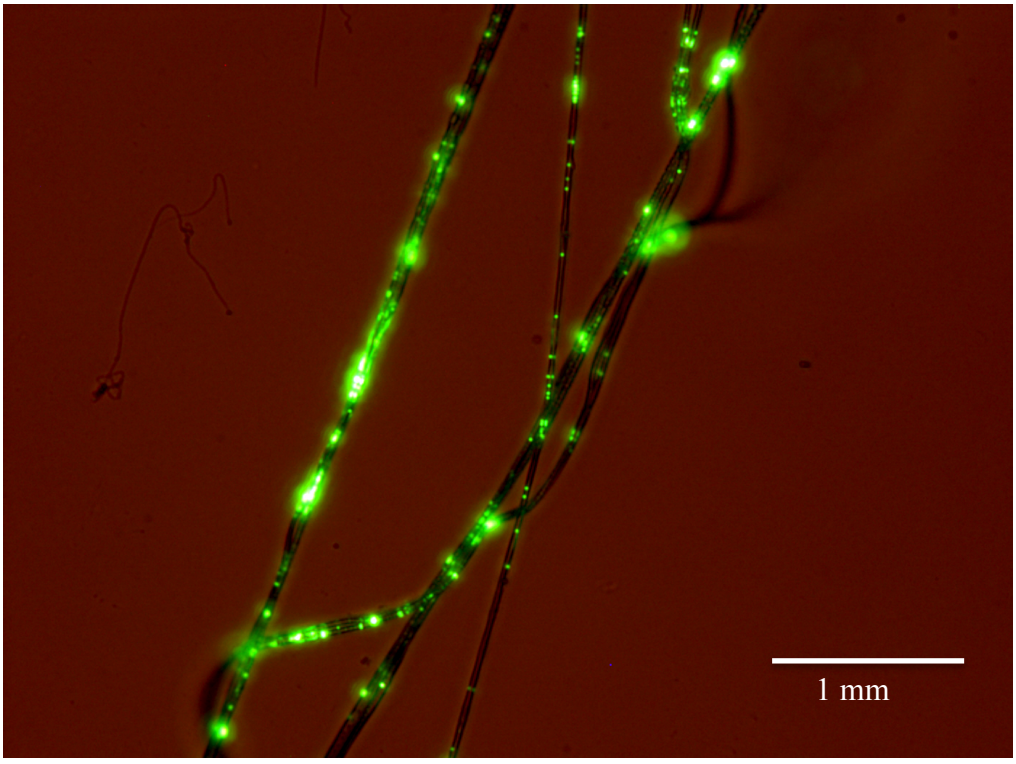


Figure 2. Living scaffold generated by way of cell electrospinning encapsulating GFP expressing N2A cells. Similar living architectures were generated by means of aerodynamically assisted bio-threading.

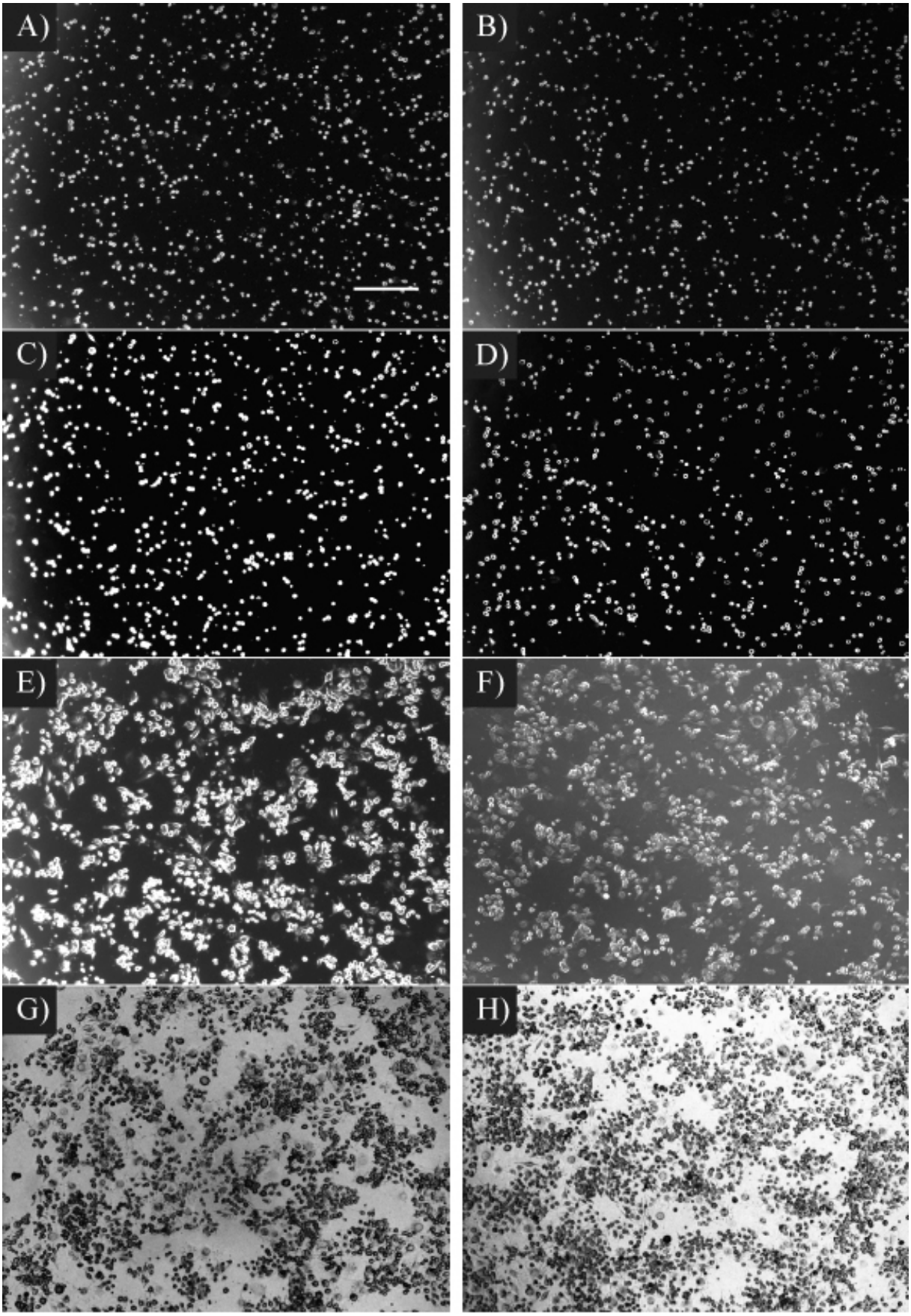

A)
B)
C)
D)
E)
F)
G)
H)

Figure 3. Characteristic optical micrographs of A, C, E, G and B, D, F, H of controls and CE samples respectively at a magnification of X4 for 0, 24, 48 and 72hrs post-treatment. Similar to the CE samples the AABT samples were indistinguishable from the controls. The scale bar in panel A represents 100μm and is applicable to all panels.

Several samples of cellular scaffolds were collected from both CE and AABT preparations and incubated in culture media at 37^{o}C and 5% CO_2 initially for at least 30 minutes. The scaffolds were subsequently put into culture medium in Petri dishes until required for analysis. Post-incubation cells were recovered from at least five samples from each group, using either cell recovery or dispase. The recovered cells were assessed over 72hrs, analyzed phenotypically and labeled for flow cytometry (Figures 3 and 4). Our studies found that treated cells did not demonstrate any phenotypic alterations (Figure 3), and were indistinguishable from controls. Flow cytometry analysis showed similar cellular viability to our previous work. Staurosporine treatment led to cells undergoing apoptosis at 24hrs (Figure 4B), as expected. Mitochondrial membrane potential was also analyzed over the same time course (Figure 4C), and correlated well with our previous *in vitro* work. Finally we used flow cytometry to analyse cellular proliferation over a shorter time course (4 to 24hrs post-treatment), in order to understand whether cellular alterations took place just after treatment by either process. These studies show that the treatment processes have no effect on cell proliferation in comparison to controls (Figure 4D). Real-time *in vivo* bioluminescent imaging of mice implanted with control, CE and AABT tumour cells demonstrated that neither CE nor AABT treatments compromised the ability of the cells to proliferate *in vivo;* both treatment groups demonstrated similar tumour growth kinetics to the untreated control cells over the course of the experiment (Figure 5).

The studies reported here demonstrate that neither cell electrospinning (CE) nor aerodynamically assisted bio-threading (AABT) have any detectable effect on the *in vitro* or *in vivo* growth of the cells. This extends our previous investigations using bio-electrosprays and aerodynamically assisted bio-jets, which were also safe in terms of growth and survival of treated cells. Composite living structures have also been found to integrate post-transfer with their hosts without causing any form of rejection. These results increase our confidence in these bioplatform technologies, which we believe will have significant ramifications for the development of three-dimensional biological models, and could also contribute to the development of pre-orientated and organized architectures for repairing, replacing and rejuvenating damaged and/or ageing tissues, whilst also adding a novel approach to the delivery of biological therapeutics.

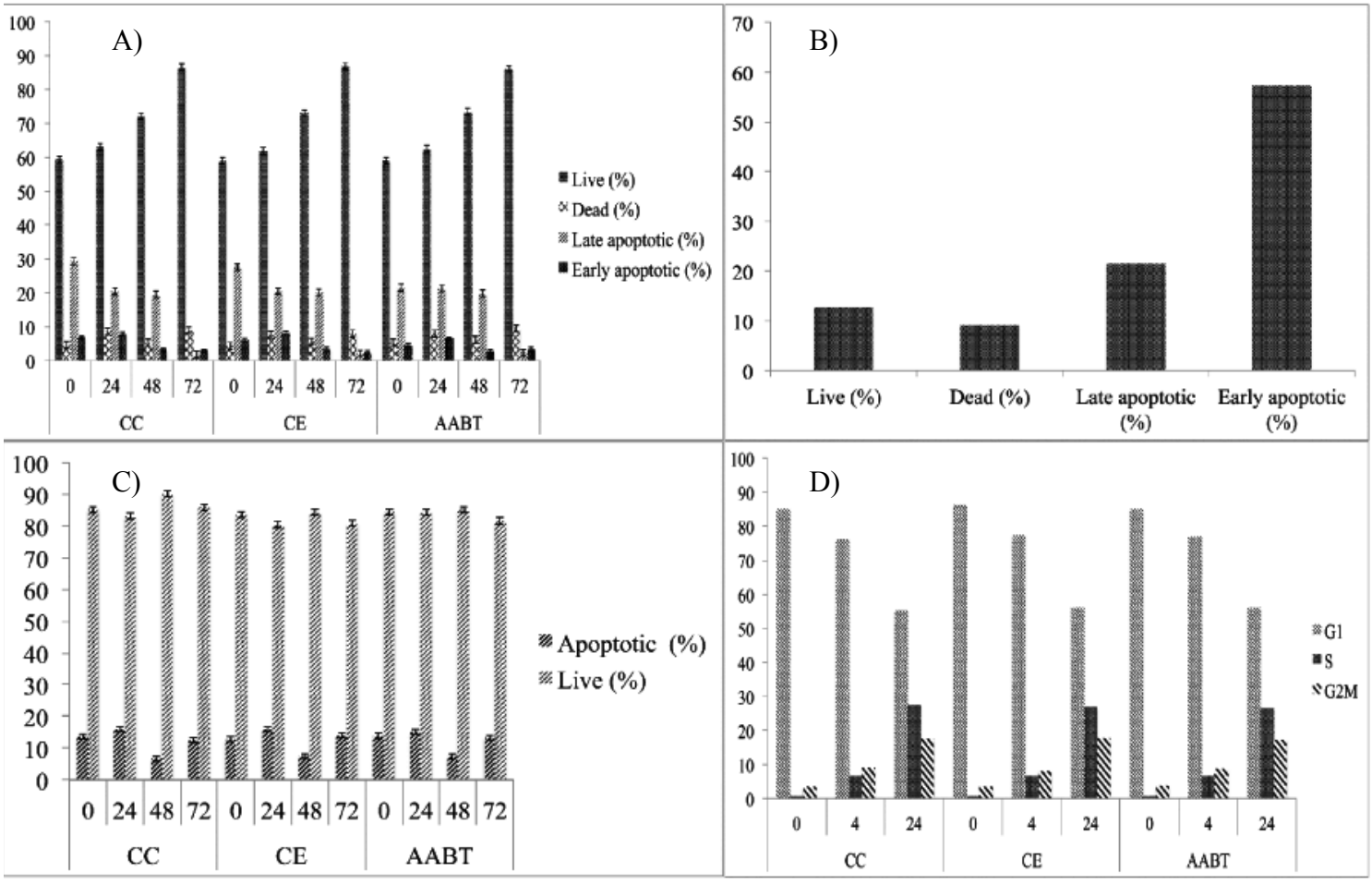


Figure 4. Representative bar charts showing A) cellular viability of post-treated cells in comparison to controls over a time course of 72hrs [n=5, SD ±5%], B) cells subjected to staurosporine and analyzed at the time point of 24hrs, C) proportion of apoptotic cells as indicated by mitochondrial membrane potential of the three samples measured over 72hrs [n=3, SD ±3%] and D) cell proliferation of the three samples assessed at 0, 4 and 24hrs post-treatment. Note in all panels the y-axis represents cellular percentage (%).

Experimental Section

The cells explored in these studies were mouse neuroblastoma cell line N2A, which have a high rate of proliferation and are able to form tumors in A/J mice. [11] These cells were cultured in Dulbecco's modified eagle medium (DMEM) (with 10% fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin) in tissue culture flasks and incubated at 37°C with 5% CO_2. For real time *in vivo* imaging purposes, the luciferase gene was introduced into the N2A cells by way of a lentiviral system based on the LNT vector type, [12, 13] rendering the cells capable of bioluminescence. [13] The tagged cells were suspended in culture medium and incubated at 37°C with 5% CO_2 until they were required for CE and AABT.

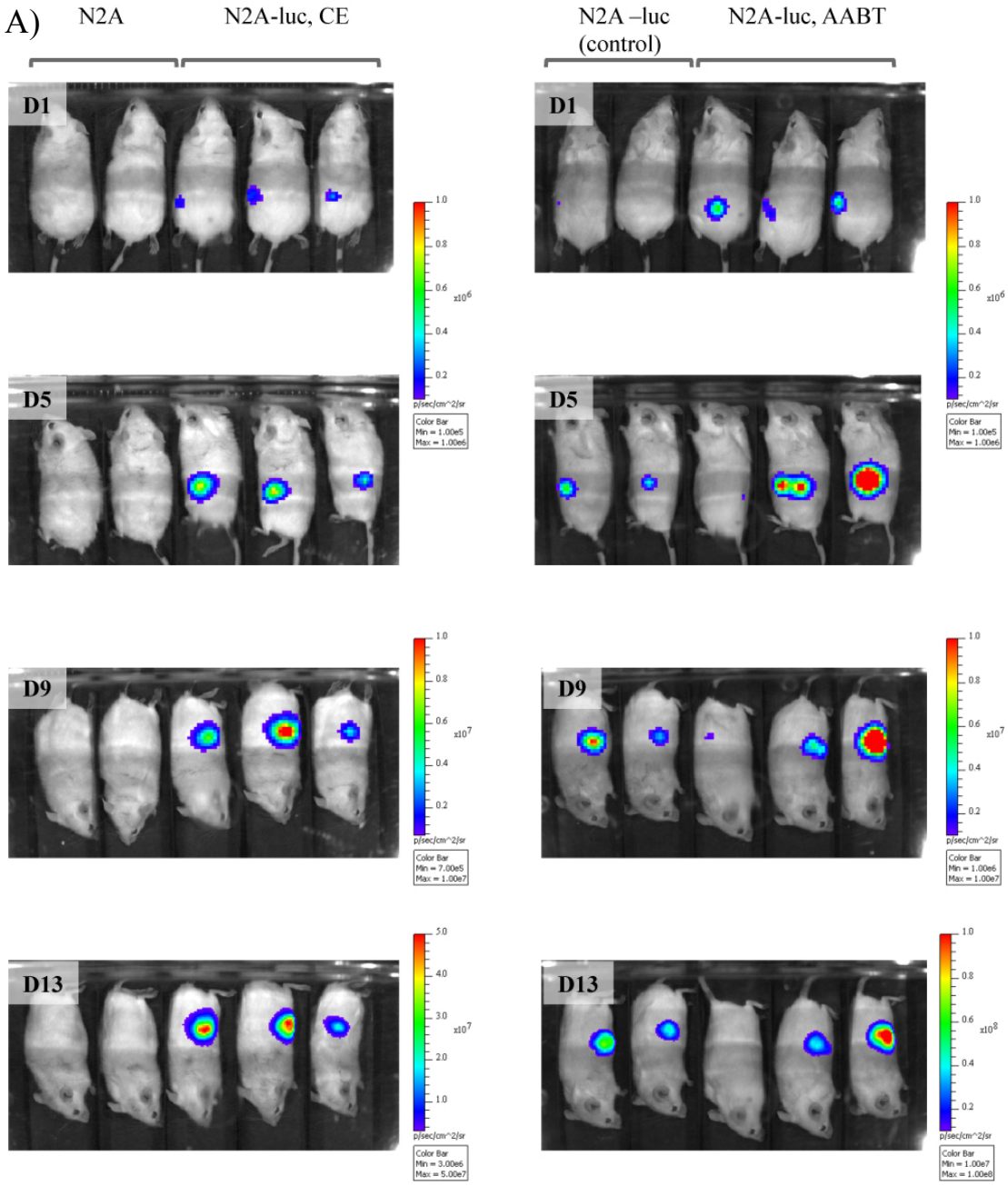
The CE system explored in these studies used inner and outer needles with diameters in the ranges of ~800µm and ~2000µm respectively with both having a wall thickness of ~300µm. Collection took place with the aid of a fine wire mesh, which was submerged in DMEM approximatly 5mm and was maintained at 37^{0}C during fiber generation. During these cell electrospinning studies the electric field varied from 0.05-0.25 kV/mm. In the case of AABT the needle utilized was a ~1500µm single needle and collection was onto a similar mesh placed ~5mm below the surface of DMEM, which was maintained at 37^{0}C. The mesh

explored in these studies for collecting the genarated fibers/scaffolds had a square mesh configeration. Each induvidual mesh had the dimensions of ~6mm x 6mm with the mesh made with a wire having a thickness of ~1mm. The biopolymer used in both spinning scenarios was modified matrigel (BD Bioscience, Oxford, United Kingdom), which had a high concentration of laminin. Matrigel and modified versions have been explored previously with cells, for transfer into living hosts by way of intraperitoneal injection.[13, 14] Those studies have demonstrated that matrigel allows the efficient exchange of nutrients between the cells within the matrigel and its surrounding microenvironment through the matrix pores, without any alterations to the cell's function. Samples were removed from the mesh and placed in Petri dishes containing Dulbecco's modified eagle medium (DMEM) (with 10% fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin). The scaffold samples generated in these experiments were namely 1) untreated control N2A, 2) untreated luciferase expressing N2A cells, 3) CE-treated luciferase expressing N2A cells and 4) AABT-treated luciferase expressing N2A cells. Several living scaffold samples were generated for each of these groups. For *in vitro* and *in vivo* studies the cells were recovered using either cell recovery solution or dispase (BD Bioscience, Oxford, United Kingdom) [14, 15]. Breifly the cell recovery process was carried out by immersing the scaffold samples into either solution (cell recovery or dispase) which over ~15mins was seen to desolve and disassociate the cells from the scaffold. These cells were then centrifuged into a pellet and resuspensed in PBS (Phosphate Buffer Saline). The cells were washed three times in PBS before they were explored for *in vitro* and *in vivo* studies in these investigations. Prior to starting our flow analysis we phenotypically assessed the cells using a Leica phase contrast microscope over 72hrs (Figure 3).

For *in vitro* analysis we used flow cytometry to assess cell viability (Dead cell apoptosis kit, Life Technologies, V13242), mitochondrial membrane potential (Life Technologies, V35116) and cell proliferation (BD Biosciences, 347583). Assays were performed according to the manufacturer's protocols and measured using a BD LSR II (Figure 4). In each case the flow cytometry measured over 20000 events. As a negative control we employed staurosporine (Invitrogen, PHZ1271), to inhibit a variety of kinases thereby triggering apoptosis (Figure 3B).

In vivo imaging of the treated cells transferred into mice was performed in accordance with the Animals Scientific Procedures Act (1986) and approved by the local Ethical Review Committee. Six to eight week-old A/J mice (Charles River UK Ltd) were used and anaesthetized by intraperitoneal injection of 50 mg kg^{-1} body weight ketamine (Ketaset; Fort Dodge Animal Health, Southampton, UK) and 5 mg kg^{-1} body weight xylazine (Rompun; Bayer, Newbury, Berkshire, UK) to conduct both cell implantation and real time bioluminescence imaging. Mice were subcutaneously implanted in the left flank with $5x10^4$ cells in a total volume of 100 µl. Tumor growth was assessed by bioluminescent imaging using an IVIS® Spectrum system (Caliper Life Sciences, Alameda, USA) which consists of a cooled charge-coupled device camera mounted on a light-tight specimen chamber. Mice were imaged 10-15 min after intraperitoneal injection of 300 mg kg^{-1} body weight D-luciferin mixed with the anesthetics. A grey scale reference image was taken under low illumination prior to quantification of emitted photons over 0.5 s to 2 min, depending on signal intensity, using the software program Living Image (Caliper Life Sciences). A pseudocolor image representing light intensity (blue, least intense to red, most intense) was generated using the Living Image software and superimposed over the grey scale reference image.

Bioluminescence within specific regions of individual mice was also quantified using the region of interest (ROI) tool in the Living Image software program (given as photons s^{-1}). Animals were imaged at different time points over 13 days (Figure 5).



B)

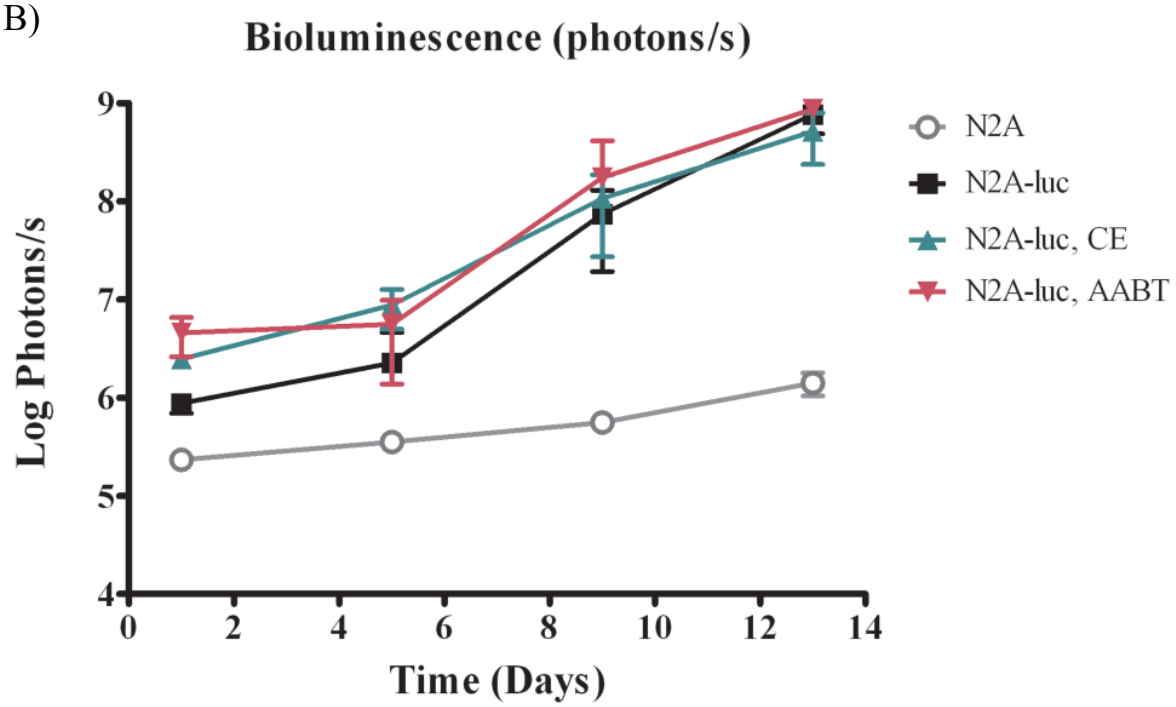


Figure 5. (A) Real-time bioluminescent imaging of mice implanted with 5 x 10^4 untreated N2A cells (labeled as N2A), untreated luciferase-expressing N2A cells (labeled as N2A-Luc), cell-electrospun luciferase-expressing N2A cells (labeled as N2A-Luc, CE) or aerodynamically assisted bio-threaded luciferase-expressing N2A cells (labeled as N2A-Luc, AABT). 300 mg kg^{-1} body weight of D-luciferin mixed with ketamine and xylazine were administered by intraperitoneal injection 10–15 min prior to image acquisition. Mice were imaged at time points 1, 5, 9 and 13 days post-implantation. Images were obtained using an IVIS Spectrum and are displayed as pseudocolor images of peak bioluminescence (given as photons s^{-1} cm^{-2} sr^{-1}), with variations in color representing light intensity at a given location. Note that the day 1 and day 5 images are shown at a different scale to day 9, and from day 13 images. (B) Bioluminescence (given as photons s^{-1}) in the left flank region was quantified using the Living image software. The values for each mouse are shown and the means and standard deviations were used to plot the graphs. A one-way ANOVA with Bonferroni

multiple comparison post-test (comparing all pairs of columns) for each time point, for N2A-luc, N2A-luc CE and N2A-luc AABT. There was no significant difference at any time point.

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Cell electrospinning and aerodynamically assisted bio-threading are novel bioplatforms for directly forming large quantities of cell-laden scaffolds for forming living sheets and vessels in three-dimensions. Functional biological architectures generated as those shown herein are most useful in both the laboratory and the clinic.

Cell electrospinning and aerodynamically assisted bio-threading living cells

Cell electrospinning an *in vitro* and *in vivo* study

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