A Novel Direct Aerodynamically Assisted Threading Methodology for Generating Biologically Viable Microthreads Encapsulating Living Primary Cells

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Received 28 April 2007; accepted 14 July 2007
DOI 10.1002/app.27190
Published online 9 October 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: In a recent discovery, coaxial electrospinning was explored to encapsulate living organisms within a continuous bio-polymeric microthread from which active biological scaffolds were fabricated (Townsend-Nicholson and Jayasinghe, Biomacromolecules 2006, 7, 3364). The cells were demonstrated to have gone through all expected cellular activity without their viability being compromised. These biologically active threads and scaffolds have direct and tremendous applicability from regenerative to therapeutic medicine. Currently these post-processed cells as composite threads and scaffolds are being investigated in-depth at a cellular level to establish if the processing methodology has any affect on the cellular make-up. We now demonstrate a competing non-electric field driven approach for fabricating composite threads and scaffolds influenced only by a differential pressure. We refer to this novel composite thread to scaffold fabrication methodology as coaxial aerodynamically assisted bio-threading (CAABT). Our investigations firstly, demonstrate that this technique can process handle living organisms without biologically perturbing them in anyway. Secondly the process is elucidated as possessing the ability to form composite active threads from which biologically viable scaffolds are formed. Finally our study employs florescent activated cell sorting (FACScan), a method by which the cellular dynamics and viability are quantified on control and threaded cellular samples at two prescribed time points. In parallel with FACScan, optical comparison of cellular morphology at three time points within a period of three weeks is carried out to photographically observe any changes in the post-processed cellular phenotype. Our developmental investigations into this novel aerodynamically assisted threading methodology has unearthed a unique biomicrofabrication approach, which joins cell electrospinning in the cell threading to scaffold fabrication endeavor. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 1215–1225, 2008

Key words: coaxial aerodynamically assisted bio-threading; primary living cells; biologically active threads and scaffolds; regenerative medicine; flow cytometry

INTRODUCTION

Today's international research environments combine physics, chemistry, and biology/medicine, which have truly answered some of humankind's most interesting questions. Hence research in the 21st century is multidisciplinary, breaking down barriers finally resulting with ground breaking discoveries some of which have recently been recognized by the Nobel committees.[1] Cross-fertile research has curved out many emerging areas of research, in particular evolving an extensively publicized field which is referred to as bioengineering and biotechnology, which is widely interpreted within the scientific community. Regenerative to therapeutic medicine which are life science areas of research today are underpinned within the bioengineering and biotechnology banner. The vibrant collaboration between physicists, chemists, biologists, and clinicians have given birth to an exciting and rapidly emerging to self-standing field of research, referred to as tissue engineering. This is a research field striving to study the science, engineering, and medical aspects where viable tissues to one day fully functional organs are reconstructed for repair and/or replacement.[2]

Tissue engineering is carried out in many different methods and protocols.[3] These approaches could be categorically segregated as non-jet and jet-based

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Contract grant sponsors: The Royal Society (Seed-Corn research grant and a Wolfson Laboratory Refurbishment Grant), Engineering and Physical Research Council, UK; contract grant number: EP/D506964/1.

Journal of Applied Polymer Science, Vol. 107, 1215–1225 (2008)
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techniques. Non-jet based methods have to a greater extent been investigated and have essentially given realization to this philosophy. Although some routes are rather robust the fabricated tissue constructs have resolutions placed in the millimeter scale. Other methods in this category have harnessed the nanometer scale over large areas with methods such as soft lithography to photolithography approaches; however, some of these methods have been found to be peculiar to specific cell types, rather costly and are not economical when compared with jet-based routes, which have recently been elucidated as having a comparable resolution. In this article we will focus on jet-based tissue engineering methodologies. There are principally four different jet-based tissue engineering methodologies currently being investigated by a global scientific community. These are namely, ink jet printing (IJP),[4] laser guided cell writing[5] and very recently uncovered bio-electrosprays (BES),[6] and cell electrospinning (CE).[7]

IJP could be said to have fathered the jet-based category providing the required motivation and focus for jet-based methodologies to pursue its contribution to tissue engineering. IJP exploits the expansion of a system either thermally or by way of piezoelectricity embedded within needles, which force out media through a needle as a droplet. This drop generation technology has been coupled with computer controlled three-axes movement to fabricate structures with a wide variety of materials to the most complex namely living organisms. Several different complex architectures, which are biologically viable have been fabricated harnessing this precision drop and place fabrication approach.[8] Although IJP has achieved great milestones in the biofabrication of active tissues, the technique is hampered by the processable cell concentration in suspension. This is directly a result of the technology itself which governs the cell-bearing droplet residue sizes in relation to the employed ink jet needles. Practically the droplet sizes are double the size of the used needles, it must be noted that these droplets spread on deposition. Generally IJP uses needles sized in the 30–60 μm range, which generate droplet residues ≥100 μm. Hence using such fine needles limits the cell concentration, which if exceeded promotes needle blockage to the increase in cell debris because of the high shear forces subjected on the cells while in the needle. Although IJP has its drawbacks it is important to recognize that it has paved the way to jet-based tissue engineering. Laser guided cell writing is an approach where finely tuned lasers are used for guiding cells to pre-determined destinations on a substrate. This technique is most interesting as it achieves this by the placement of single cells one-by-one. In a material engineering perspective it could be said that tissues fabricated with this technology would have limited structural defects, but on the other hand the technique is not economical when the need is for fabricating large and complex architectures. Having said that this route is being widely explored by several biologists where investigations are carried out to understand cellular interactions between neighboring single cells to cells within a fabricated tissue.[9]

Contrary to the earlier two approaches electrosprays and electrospinning[10,11] are primarily driven by the charging of media within a conducting needle, which is later subjected to an electric field. The former initiate's droplets with controlled jet break-up while the latter forms uniaxial continuous threads. The generated droplets and threads by these two techniques have previously been shown to be placed in the micrometer and nanometer scale. Recently these techniques were uncovered as biotechniques having the ability to directly process fabricate living organisms and are referred to as BES and CE.[6,7] Living cells processed by these electrified techniques are currently undergoing an extensive investigation for any changes if any at a cellular level for identifying any cellular make up alterations. It is important to note that these techniques unlike IJP have no needle-droplet relationship implying that a nano sized organism (for e.g. bacteria) could be either contained within a droplet or thread sized in the nano-remit (<50 nm).

In this article we introduce a novel and direct biothreading technique which is only influence by a pressure difference over an exit orifice. The technique employs a coaxial needle configuration embedded within a chamber having another input for a gas. The needles are centralized with an exit orifice, which is placed directly below. The gas input gives rise to a differential pressure within the chamber with respect to its surroundings. This pressure differential draws out the media from the coaxial configuration subsequently promoting jet break-up to continuous threading where compound threads (not shown previously) are generated. This technique has only been previous explored for liquid–liquid[12] and liquid–gas[13] based systems. Hence this methodology has previously never been explored for processing a living cellular suspension containing viable cells.

We show here for the first time that using a coaxial needle configuration where the inner needle accommodates the flow of a primary living cellular suspension (in our study presented here we have investigated two primary cell types) while the outer holds the flow of a high viscoelastic medical grade polydimethylsiloxane (PDMS) medium, we are able to fabricate continuous threads having within them viable living cells in cell media to the formation of active biological scaffolds. Our findings have direct and many applications in biology and the medical

remit. Furthermore, we have, here introduced a non-electric field driven threading to scaffold generating methodology which will directly compete with CE.

EXPERIMENTAL

Formulation of the primary cell suspensions

Primary porcine vascular and rabbit aorta smooth muscle cells (gifted in-kind by Dr Stephen Hart, Institute of Child Health, UCL, London), were prepared using the explant method and identified by staining with an anti-smooth muscle actin antibody (Dako, UK). Both the primary porcine vascular and rabbit aorta smooth muscle cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% foetal calf serum (Life Technologies, UK) and 1% penicillin/streptomycin.

To prepare both the primary porcine vascular and rabbit aorta smooth muscle cells for threading, the cells were washed with phosphate buffered saline (PBS, Sigma aldrich, UK) followed by the addition of 0.5% trypsin/EDTA (Invitrogen, UK) at approximately 2 mL per 75 cm^2 of cellular monolayer. The cells were incubated in the trypsin/EDTA for 10 min at 37°C and 5% CO_2. The loosened cells were collected and then pelleted by centrifugation at $1000 \times g$ for 5 min. The supernatant was removed and the pellet resuspended in growth medium. Soon after the cell suspensions were prepared they were characterized for there viscosity, surface tension, to density. The cell concentrations in either of the prepared porcine and rabbit cellular suspensions were estimated using a haemocytometer. The formulated ready to thread primary cell suspensions were at a cellular concentration of 10^6 cells mL^{-1}. For residue analysis proposes similar cell loaded suspensions were prepared but had the cells and the medical grade PDMS media labelled with florescent dyes.

Coaxial aerodynamically assisted bio-threading equipment set-up

The equipment (Fig. 1) setup used for coaxial aerodynamically assisted bio-threading (CAABT) in these investigations are similar to those described previously[12,13] and consists of a coaxial needle firmly held in a glass vessel [Fig. 1(a,b)] having directly below and in-line with the base of the needles an exit orifice. The vertical needle in Figure 1(b) shows the capillary accommodating the flow of either cellular suspension which flows through medical grade silicone tubing connected to a syringe holding either cell suspension. Similarly the right hand horizontal needle in Figure 1(b) depicts the capillary accommodating the flow of the PDMS medium. The capillary on the left holds the gas flow giving rise to the differential pressure. The two individual syringes (holding either cellular suspension and PDMS media) are placed in two separate syringe pump cradles capable of delivering flow rates as low as 10^{-20} m^3 s^{-1} (PHD 4400, HARVARD Apparatus, Edenbridge, UK). The gas providing the pressure difference is supplied at 6 bar, which is precisely regulated by means of a precision digital controller having a resolution of ±0.01 bar. The experimental set-up was housed in a Class II laminar flow safety cabinet [Fig. 1(a)].

Operational parametric guides

As our intention was to study the threading of primary living cells without compromising there viability to the stable generation of continuous composite cell-bearing threads, it was decided to develop an operational parametric guide. This enabled the mapping of a parametric window in which stable continuous cell-bearing threads were generated while threading either primary cell suspension with a medical grade PDMS medium. Our investigations show that there are matching flow rates in either needle to applied pressures which determine both the thread size and their distribution which is most crucial if this non-electric field driven cell threading methodology is to compete with its other thread-based cell deployment approaches. Parametric operational conditions were identified by initially setting the flow rates to the minimum in the inner and outer needles and later applying a pressure to the chamber. This procedure was repeated and the resulting compound threads and their distributions were characterized to understand the threading behavior. Our investigations presented here studies an operational window of applied chamber pressure to flow rate regimes in either needle of 0.01–5 bar or 10^{-20}–10^{-7} m^3 s^{-1}, respectively. At identified operational conditions the parameters were set and only observed after $\sim$ 600 s which allowed the flow rates in either needle to the applied pressure to reach the selected conditions. This was necessary as the change in flow rates to applied pressure took time to settle to those selected operational conditions.

Cell-encapsulating thread residue collection and microscopy

Analysis of the coaxial aerodynamically assisted bio-threaded cells were carried out following collection of the residues on either glass microslides, on special hydrophobic polyvinylidene difluoride (PVDF) membranes (Amersham Hybond™-P, GE Healthcare, Buckinghamshire, UK) or in cell flasks. The former was carried out to primarily observe the generated thread residue distribution whilst collection on the

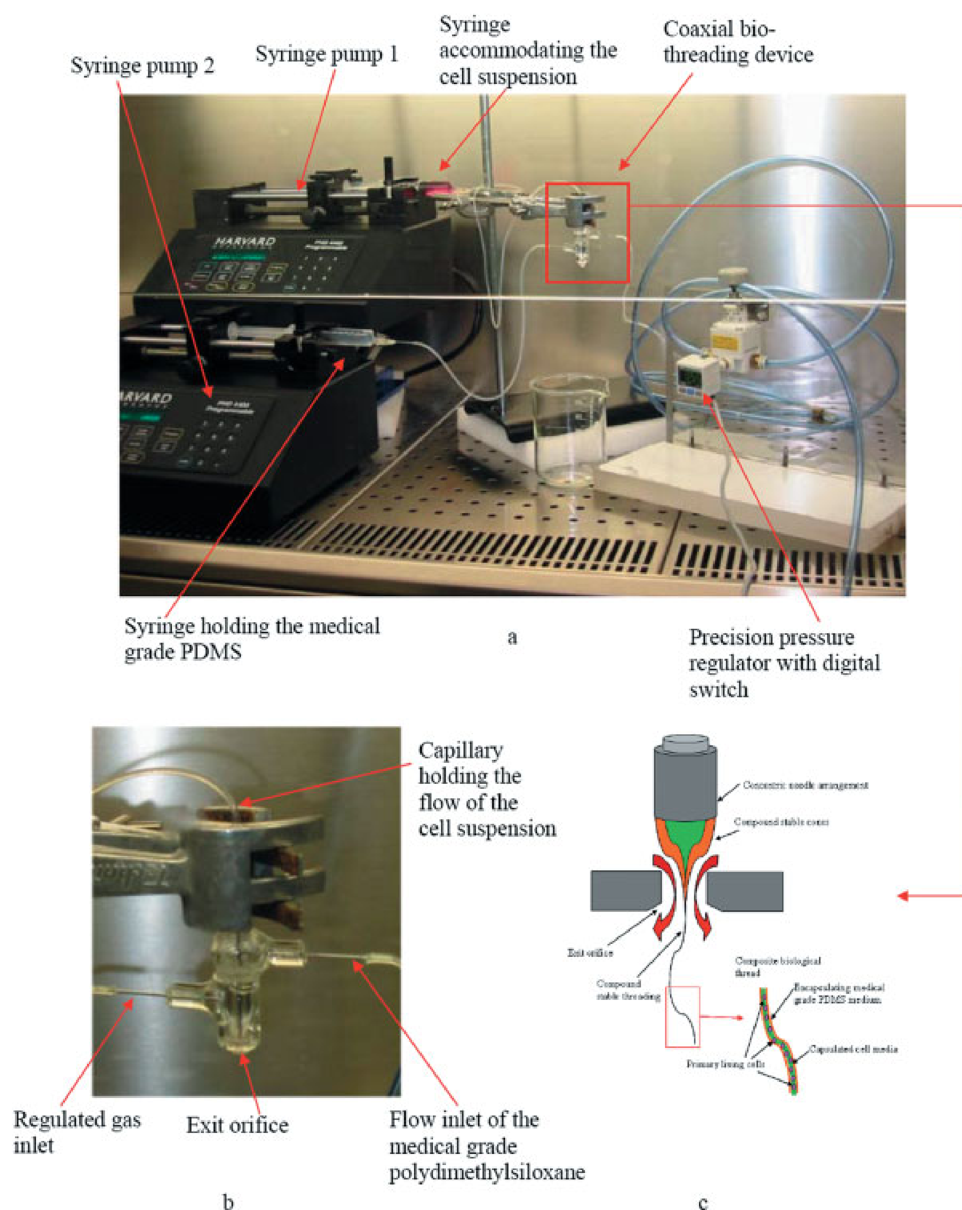


Figure 1 Digital images of (a) the coaxial aerodynamically assisted bio-threading equipment set-up housed in a Class II safety hood, (b) depicts the CAABT device, and (c) a schematic representations of the threading process. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membranes were to fluorescently observe the capsulated cells within threads to finally studying cellular viability. Several repeated measurements for collected cell-bearing residues to controls (cell suspension + PDMS medium collected via the exit orifice without the presence of an applied pressure) were collected and examined over three weeks. The non-fluorescent cell-bearing residues were optically examined using a Leica MZ12-5 microscope (Leica Microsystems, Milton Keynes, UK) with a 1× magnification tube while the labeled cellular suspensions were studied employing a Zeiss Axiovert florescent microscope. Cell confluence in the controls and threaded samples were achieved at similar times, during this time cell growth and phenotypic properties were photographically recorded at selected time points post-threading of either cellular suspension.

FACScan analysis for assessing cellular viability and dynamics

Fluorescence-activated cell sorting (FACScan) is a flow cytometry method for quantifying cellular features by optical means. Briefly, the FACScan technique and system employed can quantify both necrosis (general cell death) and apoptosis (programmed cell death). In programmed cell death the membrane phospholipid phospatidylserine (PS) is

TABLE I
Principal Physical Properties of the Media Investigated in These Studies

Sample	Viscosity (mPa s)	Surface tension (mN m^{-1})	Density (kg m^{-3})
Porcine vascular smooth muscle cellular suspension	~ 23	~ 53	~ 910
Rabbit aorta smooth muscle cellular suspension	~ 26	~ 51	~ 913
Medical grade PDMS	12,500	21	970

translocated from the inner to the outer layer of the plasma membrane. During the initial stages of apoptosis the cell membrane remains intact, whilst at the moment of general cell death the cell membrane loses integrity and becomes leaky to the vital dye propidium iodide (PI). Staining with annexin V, which has a high affinity for PS in conjunction with PI, allows the identification of live cells (annexin − PI −), early programmed cell death (annexin + PI −), general cell death (annexin + PI +), and cellular debris (annexin − PI +) to give a accurate measurement of the dynamics of cellular death.

Individual cell suspensions were prepared from either cellular suspension for control and threaded samples. Cells were mixed with Annexin V FITC (final concentration 1 μg/mL) (Pharmingen, UK) and propidium iodide (PI) (final concentration 1 μg/mL) (Sigma, UK) in the presence of 1.8 m*M* calcium. Cells were incubated at room temperature for 15 min prior to quenching in calcium containing binding buffer (Pharmingen, UK) and were analyzed immediately. A Dako Cytomation CyAn ADP flow cytometer was used to collect 20,000 events. The excitation was at 488 nm using an argon laser, the FITC emission was collected with 525 ± 20 nm band pass filter and the 7AAD with a 675 ± 20 nm filter. The data were analysed using Summit 4.3 software (DakoCytomation, UK). Thus in our studies the controls and threaded samples from either primary cell suspensions were passed though the FACScan apparatus to analyse the number of live and dead cells through to cells, which were undergoing apoptosis.

High-speed imaging of cellular threads

A high-speed digital camera capable of capturing 160,000 fps (Phantom V7.1, Photo-Sonics International, Oxford, UK), was set in-line and in conjunction with a range of Nikon and Leica lens (Nikon Macro NIKKOR 25–85 mm and Micro NIKKOR 105 mm and Leica Mono Zoom 7, UK), arranged along with a light source (Solarc® Metal Halide, 60 W, Everest VIT Part No. SPL-600). When the camera was triggered it recorded the threading process in real time from exit orifice to well below. The high-speed image sequences were recorded directly into a personal computer. These captured high speed images were calibrated accordingly to observe the threading characteristics.

RESULTS AND DISCUSSION

Cellular suspensions and CAABT

Specially formulated cellular suspensions either containing living primary porcine vascular or rabbit aorta smooth muscle cells were characterized individually together with the medical grade PDMS medium (used for capsulation). Table I summarizes these measured and estimated values, which illustrate that there is very little difference in terms of the media density. However, the media properties viscosity and surface tension of the PDMS in comparison with the cell suspensions are three orders of magnitude larger and approximately half that of the cellular suspensions, respectively. Unlike in our previous studies with BES[6,14,15] and CE[7] the electrical conductivity of the cellular suspensions and the PDMS media have no effect on this technique. However, in aerodynamically assisted bio-threads viscosity together with the media rheological properties in combination with the flow rate of the media to the needles and the applied chamber pressure are the governing properties of this threading methodology. Till recently these coaxial aerodynamically assisted methodologies were only investigated for single phase liquids in some cases in combination with a gas. Hence the use of this coaxial threading methodology to process handle living organisms is novel.

This first example of CAABT of living primary cells was carried out for an operational parametric space of 0.01–5 bar and 10^{-20}–10^{-7} m^3 s^{-1} of applied chamber pressure to a variety of combinations in flow rates in either needle, respectively. As we needed to establish stable threading conditions, where the thread was repeatedly seen to promote the generation of a continuous composite thread several flow rate combinations in the inner to outer needle to applied pressures were investigated within the identified parametric space. Our study into finding these conditions demonstrated that for a given flow rate in either needle if the pressure was too low the media flowing within the needles would be drawn out and periodically threaded implying an

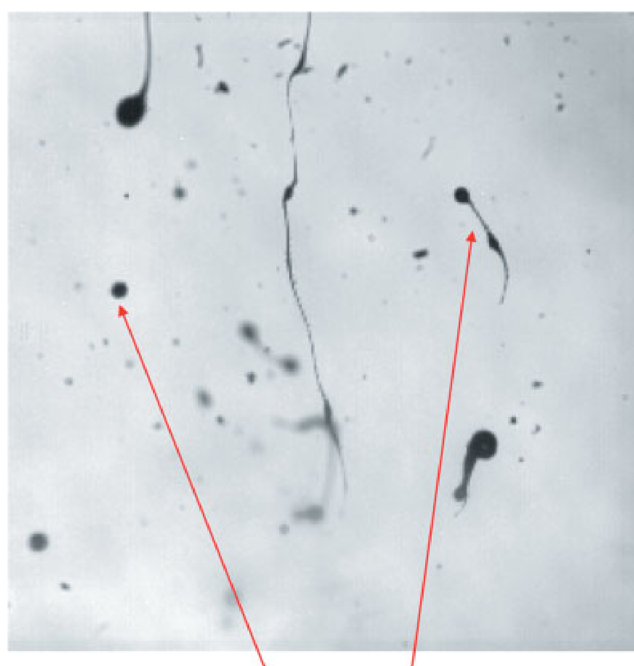


Figure 2 Characteristic high-speed image of the generated mixture of composite elongated droplets and thread fragments seen to exit the device for a non-matching applied pressure as applied to the chamber with respect to a low flow rate combination at the needles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

unstable condition. This essentially gave rise to the production of both a mixture of a polydispersed distribution of composite elongated droplets (Fig. 2) and finally thread fragments and residues, which are not desirable. Hence the flow rates in either needles were adjusted and the pressure was applied to the chamber. On observing the exit orifice region for a flow rate in either needle in the $\sim 10^{-9}$ m^3 s^{-1} regime for an applied chamber pressure of $\sim$ 0.5 bar the jet was seen to stabilize forming a continuous composite thread (Fig. 3). Magnified image sequences captured by means of high-speed photography at both the exit orifice and near collection elucidated stable continuous composite threading was achieved [Fig. 3(b,c)]. The high speed image depicted in Figure 3(b) illustrates the uniformity in the thread whist leaving the chamber and on entering the atmosphere through the exit orifice. Figure 3(c) shows the continuous composite thread capsulating either cellular suspension near the point of collection which shows the thread having a wave-like pattern. We speculate that this wave-like behavior adopted by the thread near collection is directly due to air drag imposed on the thread by the surrounding air. The subjected air drag introduces an eccentricity or an angular component to the vertically downward drawn continuous thread much like those seen in both electrosprays[16] and in electrospinning[17] which introduces a whipping or wavy behavior to the jet or thread, respectively. During these experiments we investigated the possibilities of optimising the threading process by either increasing or decreasing the applied pressure to flow rates in either needles while keeping one parameter constant. It was observed that in either case there existed a critical point for that given parameter. On keeping the flow rates constant and increasing the applied pressure, before reaching the critical point the threading process was observed to show the generation of multi-continuous composite threading where multiple threads were

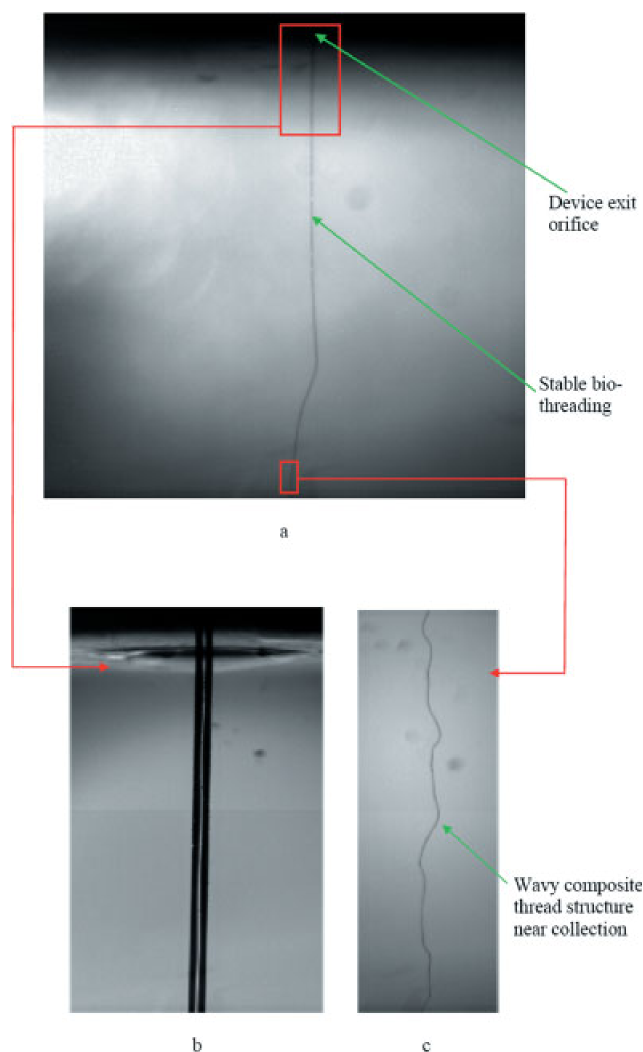


Figure 3 Representative high speed images depicting (a) stable coaxial bio-threading, (b) the uniform thread exiting the pressurized chamber via the exit orifice and (c) the wavy behavior of the compound thread directly brought on by the surrounding air imposing air drag on the composite thread near the point of collection. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

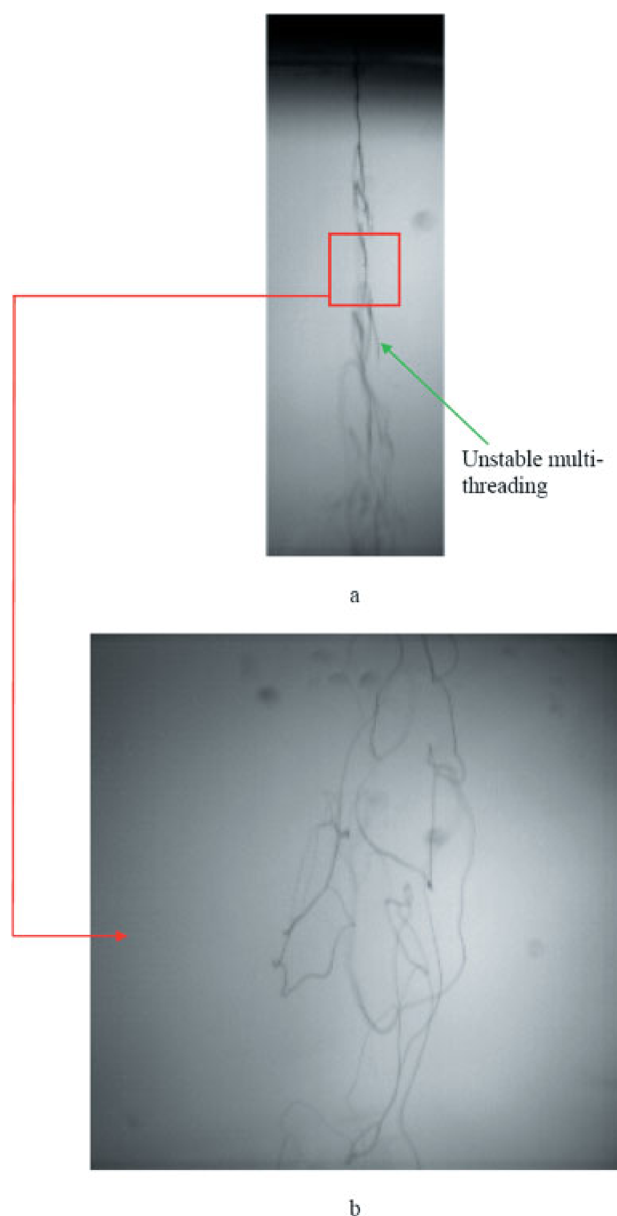


Figure 4 (a,b) Characteristic high speed images demonstrating the violent coaxial bio-thread whipping resulting with the increase in applied pressure subjected on the coaxial thread. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

detected, we believe these result as the thread is whipping violently (Fig. 4). On further increasing the applied chamber pressure high-speed imaging elucidated the generation of a mixture of a myriad number of composite droplets and thread fragments much like that observed in Figure 2. However at extreme applied pressures the loss in capsulation was noticeable, which is a direct result of the media properties. On increasing the applied pressure even further was shown to either seize the jetting/threading process to instigating the flow of air into the syringes, which completely hinders the process.

While threading in stable operating conditions several glass microslides were swiftly moved across the threading region which collected the cell-bearing threads. Optical microscopy carried out on these residues demonstrated that thread diameters varied from $\sim$ 50 to $\sim$ 350 μm with a mean of approximately 185 μm. We also noticed that within threads cellular clusters were formed resembling a bead capsulated by the PDMS media and these cellular beads randomly varied in size (containing a varied cellular population within) along the entire length of the thread. The non-uniformity in the capsulated cellular clusters, are hypothesized to have resulted from either the significant differences in the properties of each cellular suspension when compared with the medical grade PDMS or may have risen from the random aggregation of cells while in suspension. The authors are currently vigorously pursuing many avenues to identify the reason for this behavior. A representative bio-thread examined by means of florescent microscopy (Fig. 5) demonstrated a variation in capsulated cellular populations as cellular beads along the thread. Exposing the substrate to a longer time to the bio-threading processing gave rise to a bio-scaffold much like those previously observed.[7,18]

Cellular viability post-CAABT

In investigations such as these ascertaining cellular dynamics is fundamental as the progression of the threading protocol hinges on this very element.

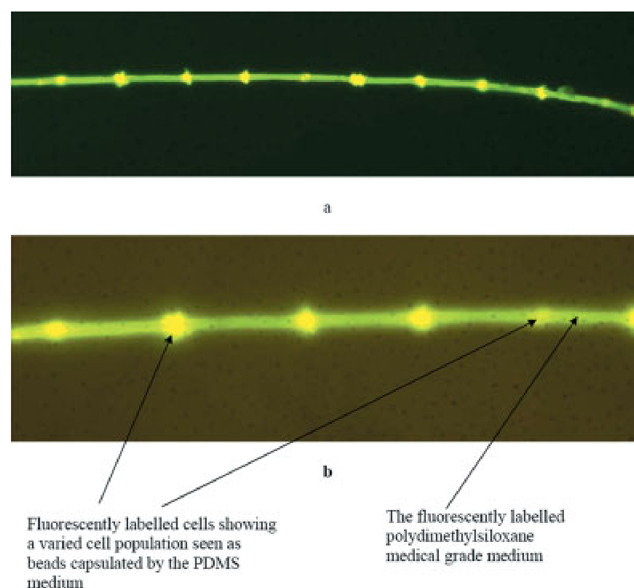


Figure 5 Representative florescent micrographs of collected residues (a) composite bio-thread and (b) a magnified micrograph demonstrating the random cell populations capsulated as beads along the bio-thread, while threading in stable operational conditions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

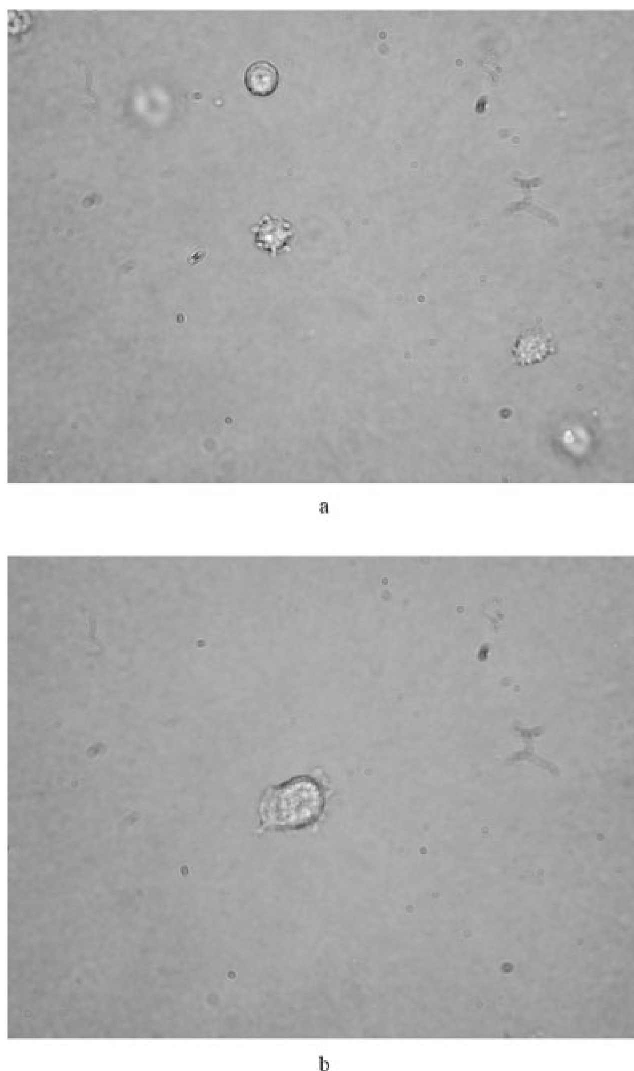


Figure 6 Typical optical micrographs depicting the collected (a) control cellular samples (without the application of an applied pressure) and (b) threaded samples during stable operational conditions. These micrographs depict the porcine cells which were very similar when compared with those collected for respective samples for controls and threaded for rabbit cells.

Therefore investigating cellular viability was addressed by employing a fluorescence-activated cell sorting methodology. Several controls (which were passed thought the threading device without the application of an applied pressure difference) and threaded samples were assessed by means of this flow cytometry approach. Figure 6(a) shows a representative optical micrograph of collected control porcine cells via the device (which look very similar to the rabbit control cells). The threaded cells (for both primary cell types) in comparison with the controls appeared morphologically similar [Fig. 6(b)]. These observations were made for several collected and threaded samples, which clearly demonstrated that either collected cells failed to show any signs of cellular trauma post-collection. Figure 7 demonstrates by means of a bar chart the flow cytometry statistics for 1, 2 porcine vascular and 2, 3 rabbit aorta control and threaded cells, respectively. The flow cytometry statistics for day 0, (Table II and Figure 7) revealed the large population of cells that are living and those that are undergoing apoptosis and necrotic. During flow cytometry, samples were gated to exclude cell clumps and cellular debris by their relative size as measured by their front shift and side shift characteristics. The remaining cells were analysed for the apoptotic and necrotic markers as measured by annexin FITC and PI respectively. Analysis of the threaded primary cells soon after collection are seen to be very similar to each other and when compared with their representative controls (as seen from Table II and Figure 7). The percentages of viable cells (Table II) post-threading when compared with their controls demonstrate that in both controls and as threaded on day 0, at least 64% of cells remain viable, which is an attractive feature.

Post-collection (day 1) the cells in all samples were observed to retained the ability to adhere to plastic and began to proliferate. Approximately a week later the controls and threaded samples were visually examined and were found to behave as expected which showed positive cellular growth in all samples (Fig. 8). Controls for both the porcine and rabbit cells appeared to behave alike [Fig. 8(a)]. Correspondingly on comparing both the threaded primary cell type they seemed to be morphologically comparable [Fig. 8(b)] and had positive growth.

At the three week time point both collected controls and threaded samples were reassessed for their

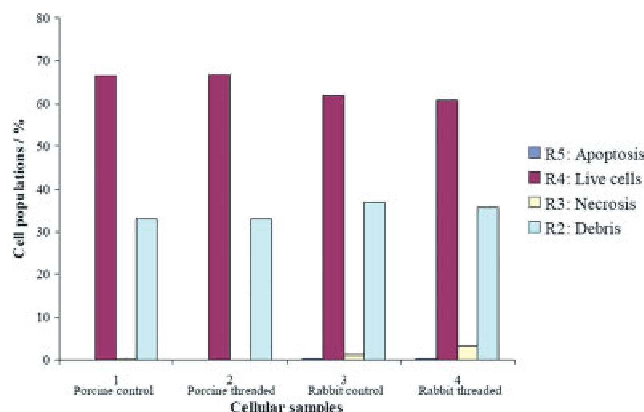


Figure 7 Characteristic bar chart plotting the flow cytometry statistics for day 0, demonstrating the respective populations of cells for 1, 2 porcine vascular 3, 4 rabbit aorta SM Cells as controls and threaded respectively. These results show an average cell viability of 64%, which is attractive. The cell population profiles for both control and threaded cells followed a similar trend (see Table II). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II
Flow Cytometry Statistics on Cell Viability at Two Defined Time Points

Sample	Collected control (without the application of an pressure differential) %		Coaxially threaded (CAABT) %	
Porcine Vascular				
Time point	0 days	21 days	0 days	21 days
R5: Programmed cell death (apoptosis)	0.08	0.98	0.07	2.20
R4: Live cells	66.60	95.58	66.84	94.62
R3: General cell death (necrosis)	0.14	0.70	0.08	1.33
R2: Nuclear debris	33.18	2.74	33.01	1.85
Rabbit Aorta				
Time point	0 days	21 days	0 days	21 days
R5: Programmed cell death (apoptosis)	0.27	10.96	0.26	2.98
R4: Live cells	61.83	86.33	60.75	95.88
R3: General cell death (necrosis)	1.13	1.50	3.28	0.39
R2: Nuclear debris	36.77	1.21	35.71	0.75

cellular viability by way of flow cytometry. From Table II and Figures 9 and 10 (flow cytometry plots) it is clear that this direct bio-threading protocol does not significantly increase either the apoptotic or necrotic cells when compared to those viable cell populations. Figure 9(a,b) depict the control and threaded porcine vascular cells, respectively, these were morphologically very similar to those rabbit aorta cells. Figure 10 elucidated by way of flow cytometry plots the cellular populations viable and those that are apoptotic and necrotic to debris for the porcine and rabbit control and threaded cells respectively for the 21 day time point [in Fig. 10(b,d, and f), quadrants R5, R4, R3, and R2 denote programmed cell death (apoptosis), live cells, general cell death (necrosis), and cell debris, respectively]. All collected cellular samples starting from approximately a 30–40% confluence, managed to reach 100% confluence at approximately the same time. Our studies presented here confirm that threaded cells manage normal growth over long periods of time post-bio-threading and do not show any long term cellular deteriorations when compared with there controls. An interesting event we noticed on observing several control and threaded samples were that controls were seen to populate cells as near-annular mono-layered cellular colonies while the threaded cells made mono-layered cellular tracks. In addition the collected threaded cells both the porcine and rabbit SMC's were growing directionally when compared with their controls. We speculate at this time that this maybe a direct result of the PDMS encapsulated threading protocol which capsulates the cells as a composite thread, promoting the direction of cellular growth. The authors are currently exploring other bio-compatible polymers which could be used for encapsulating these primary cells. As in the case of CE we are now putting these threaded cells through a study where their gene expression will be examined to understand thoroughly if there is any

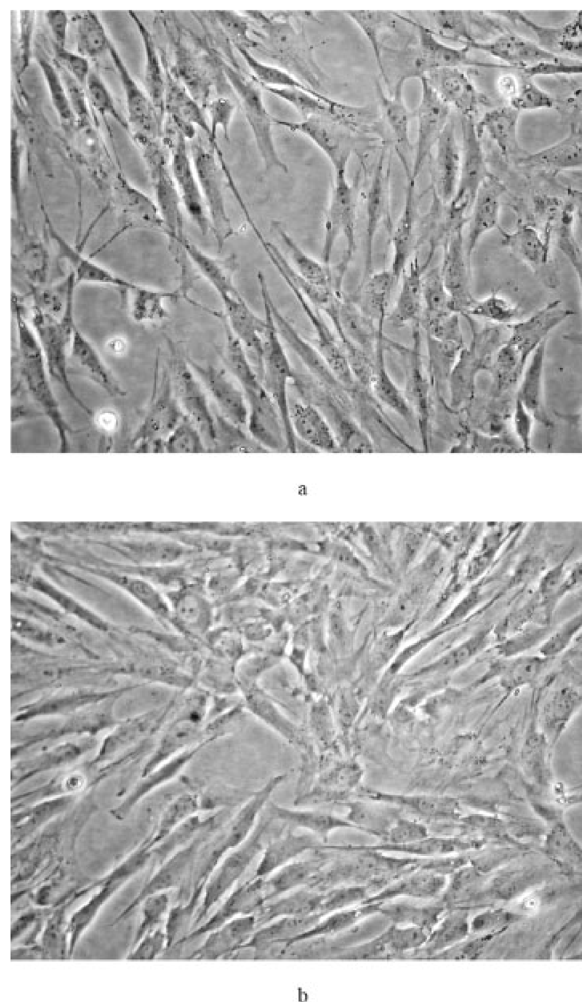
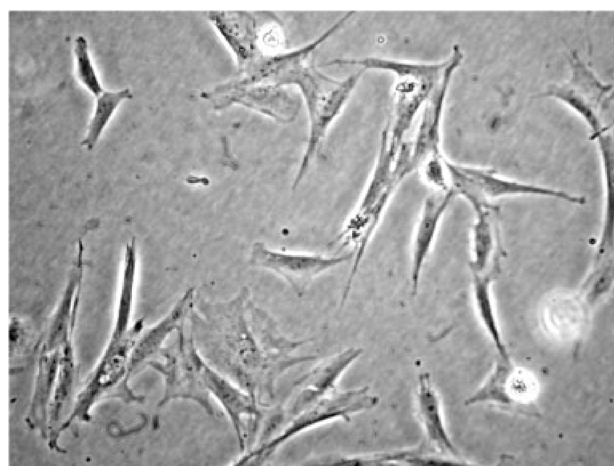


Figure 8 Distinctive optical micrographs illustrating the collected porcine (a) control and (b) threaded cells after 7 days (~ 168 h). These images are very similar to those of the controls and threaded samples for the rabbit cells, which were put through the same protocol. These micrographs also illustrate that the cells have not incurred any cellular damage via this threading methodology as they maintained their ability to adhere to the base of the cellular flasks to positively proliferate.

changes occurring to these threaded cells at a cellular level (e.g. at a chromosomal, DNA to RNA level).

CONCLUSIONS

Our developmental studies into CAABT has shown for the first time that this threading approach possess the ability to safely handle primary living cells as capsulated continuous and viable threads and residues. The threaded composite primary living cells have been compared for cellular viability with both control cells which were collected via the device without the presence of an applied pressure. The results demonstrate that this novel cell threading methodology is capable of generating cell-bearing threads for deposition to the generation of biologically viable scaffolds. The authors are currently pursuing in finding answers to the non-continuous cellular populations within threads to the coupling of this threading methodology with a three-axes plotter-like device for controlled continuous composite thread deposition for the fabrication of pre-arranged structures/scaffolds. The use of medical grade PDMS media is both ideal for capsulating such primary biological materials and is most useful for providing the required bio-compatible binding agent when these capsulated threads are deposited on one another, assisting in upholding structural integrity during the forming stage of a biological construct. The binding agent could be changed with ease to



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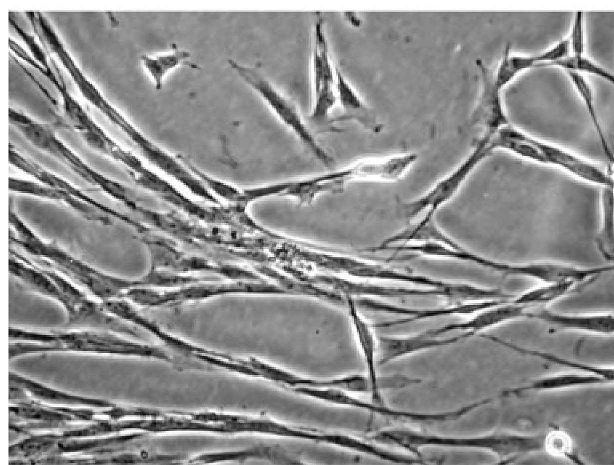

b

Figure 9 Representative optical micrographs illustrating the collected porcine (a) control and (b) threaded cells after 21 days ($\sim$ 504 h). These micrographs when compared with those for the rabbit cells were very similar. The micrographs also exemplify the fact that these cells having undergone this threading protocol have retained the ability to function as expected.

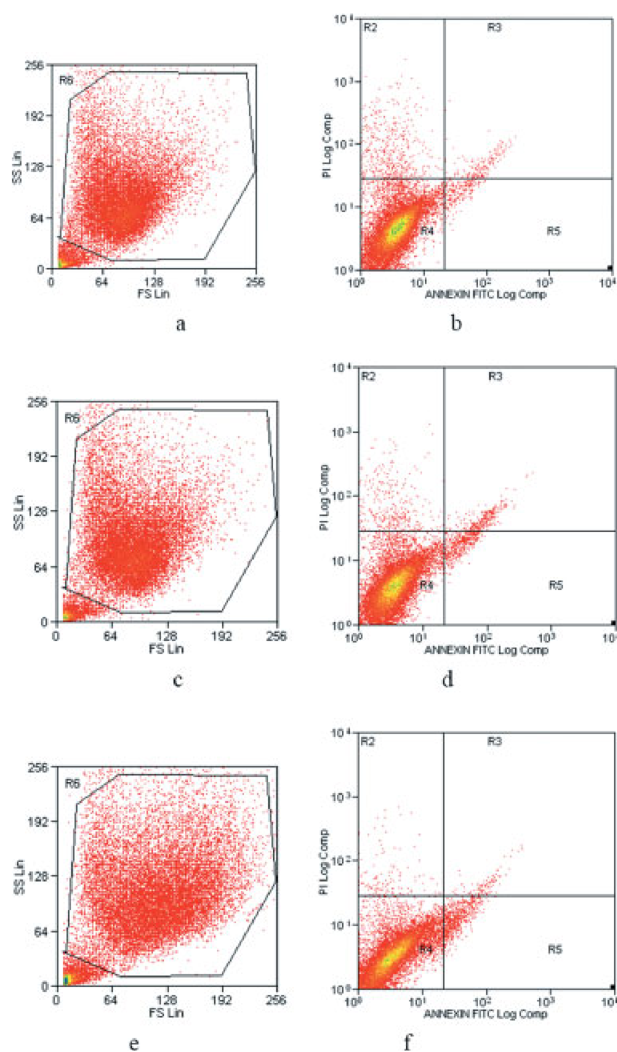

Figure 10 (a) Typical flow cytometry plots of collected (a,b) controls and (c,d) threaded samples of porcine vascular cells after a culture period of $\sim$ 21 days ($\sim$ 504 h). Plots (e) and (f) depict the respective cell populations for the threaded rabbit aorta SMCs at this time point. The porcine control plots (a and b) are very similar to those of the rabbit cells (see Table II). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

any biopolymer for e.g., collagen to chitosan. In our hands CAABT will undergo a rapid developmental program where a wide variety of living organisms will be threaded and assessed for the fabrication of tissues/constructs to one day complex biologically viable organs. This bio-threading technique has enormous direct implications to the life sciences in tissue engineering (fabricating unspecialized tissues with stem cells) to targeted cellular delivery (carried out with transfected cells in combination with gene therapy) which are a few examples. CAABT has now joined the biological-threading enterprise.

The authors are specially thankful to Mrs. Joana Sinclair of the Molecular Immunology Unit at the Institute of Child Health at UCL for all the assistance with the FACScan analysis carried out in these investigations. Mr. Adrian Walker manager of the EPSRC Engineering Instrument Pool is thanked for loaning the visualization equipment used in these studies.

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