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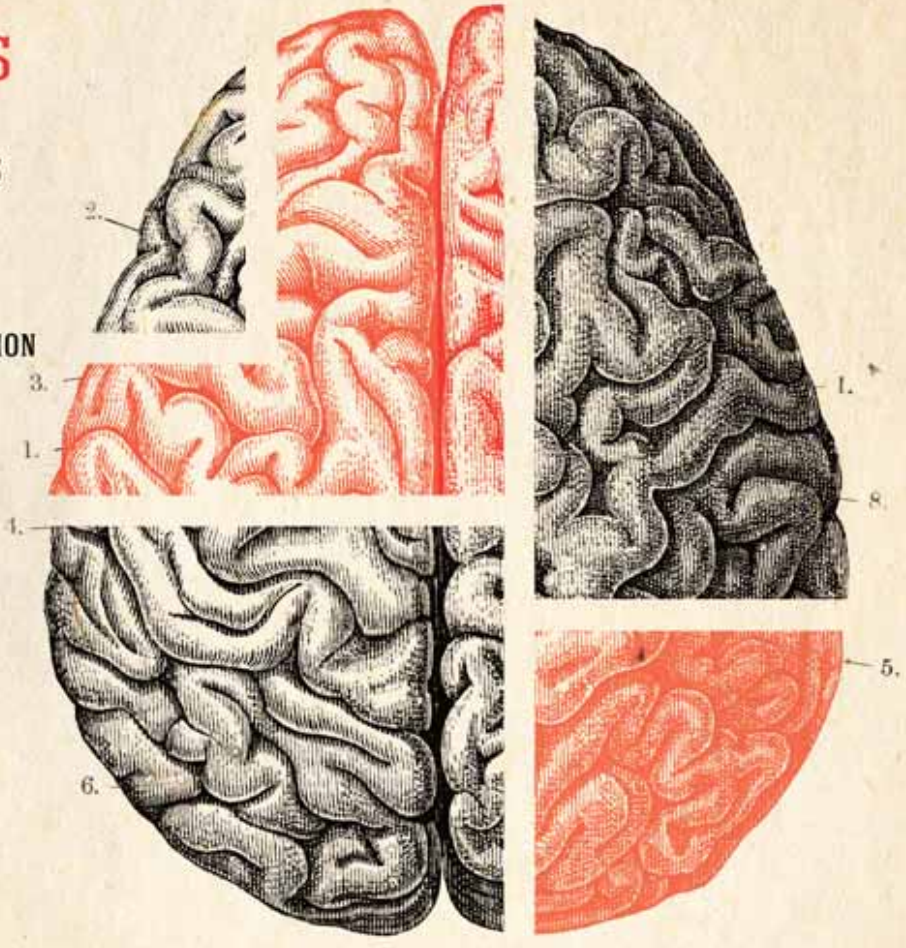

RESEARCH SPOTLIGHT

US ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES · UNIVERSITÉ LAVAL RESEARCH!AMERICA · YORK UNIVERSITY · INSTITUT BAULIEU · UNIVERSITY OF BRITISH COLUMBIA CENTRE FOR INFORMATION BIOLOGY AND DNA DATABANK OF JAPAN

Extracellular scaffolds for nervous system rehabilitation

By exploring nanophysical properties of the synthetic extracellular environment, **Professor Virginia Ayres** has uncovered some exciting applications for tissue scaffolds, verging on an innovative method for intervention following brain and spinal cord injury

Can you give a synopsis of your latest research?

My studies are looking at neural cell responses to the nanophysical properties of their external environment. We have been experimenting with a synthetic tissue scaffold to assist healing following a traumatic injury to the central nervous system (CNS: brain and spinal cord). The synthetic tissue scaffold encourages cells to grow back into a wound site and re-establish their original system sufficiently well to recover that system's original function.

Have you utilised any novel tools in your investigations?

One example is scanning probe recognition microscopy, which I pioneered and continue to develop. This is one of the few techniques that can provide accurate and quantitative information about nanofibrillar environments. Nanoscale elasticity measurements are taken with a correctly positioned tip, normal to the cylindrical surface, while auto-tracking along individual nanofibres. Nanoscale surface roughness measurements along individual nanofibres are taken with the nanofibre curvature correctly removed.

How will results from this study have an impact on regenerative medicine? If so, could you give an account of how this research will progress?

It will lead to a much more knowledgeable and tailored fabrication of regenerative scaffolds for CNS repair following traumatic injury. This will be achieved through identification of a fundamental set of nanoscale physical and growth factor properties that serve as the primary cues for the re-establishment of a CNS neural cell system.

CNS disruptions, caused by traumatic injuries, are among the most difficult situations for positive medical intervention. The standard treatment option involves encouraging the natural plasticity of the patient's CNS – guided by external rehabilitation exercises – to re-wire rather than to repair. This is typically a lengthy and costly intervention, whose outcome may only encompass improved quality of life rather than complete recovery.

The possibility of extending treatment options beyond rehabilitation to successful post-injury intervention involves research to address one of the critical barriers to progress in the field: mitigation of glial scar formation by reactive astrocytes. Our fundamental research into the directive effects of nanophysical cues introduced by nanofibrillar scaffolds has acquired a recent focus in this direction.

Would these findings act as a framework for investigating other regenerative cell scaffold systems?

Yes. Cell systems that are influenced by scaffold-based environments are ubiquitous throughout the body. Therefore, identification of the fundamental set of nanoscale properties that promote healthy neural cell physiology and function serve as the basis for quantitative exploration of other regenerative cell-scaffold systems. In addition to regenerative medicine, quantitative investigation of the nanoscale cues that direct cell-cell and cell-scaffold interactions will also advance such diverse fields as stem cell and cancer research.

Have collaborative partnerships been important for your success?

Yes, most definitely. In particular, working with Professor David I Shreiber and Dr Ijaz Ahmed of the Biomedical Engineering Department at Rutgers, The State University of New Jersey, enables us to combine investigations of the nanophysical environment cues with immunocytochemistry, which correlates cell responses with specific signalling cascades. Cell morphological responses are investigated in detail with atomic force microscopy but adding the signalling cascade information is key to understanding them. Our graduate and undergraduate research assistants are also fantastic contributors. The insights and hard work of the lead graduate student on this project, Volkan Mujdat Tiryaki, deserves special mention. In addition, the foundational work of my early collaborator Professor Sally Meiners from the University of Medicine and Dentistry of New Jersey – who first demonstrated the beneficial *in vivo* and *in vitro* effects of the electrospun polyamide nanofibrillar scaffolds – was crucial. Also, Professor Stanley Flegler, Centre Director and Electron Microscopy Specialist, Dr Melinda Frame, Confocal Microscopy Specialist and Dr Alicia Pastour, Transmission Electron Microscopy Specialist, all with the Centre for Advanced Microscopy at Michigan State University, have contributed key inputs.

Do you have any further projects in the pipeline?

Many. In collaboration with a new colleague, we are presently investigating a different set of comparative substrates that are all variations of electrospun nanofibres. This will refine our study of the effects of different polymer compositions, surface chemistries and curvatures. Next in line, working at the macromolecular level, is investigation of the biochemical factors expressed by reactive astrocytes that inhibit the re-growth of neurons. Are these too modulated by the nanophysical environment? From there, I hope we can study specific receptors with model building to address the question: how are neural cell responses modulated by specifc nanoscale properties?

Longer term, I would like to test the nanophysical cue techniques and analyses that we have developed in a different regenerative cell system. The liver cell system is of interest; this system has also been recently shown to respond to aspects of its nanophysical environments as directive cues. I am also interested in finding a collaborative partner specialising in clinical regenerative medicine, with whom we can begin a directive properties analysis of medical scaffolds currently in use and their outcomes. The goal is to be immediately useful whilst learning to ask the right questions, whose answers can help to transition this research from fundamental knowledge to therapeutic success.

Identifying nanoscale cues for brain and spinal cord regeneration

State-of-the-art nano-investigative techniques are enabling scientists at **Michigan State University** to link physical properties at the nanoscale with important cellular responses; ultimately offering new insights into regenerative neural cell systems utilising synthetic extracellular nanofibrillar scaffolds

PIONEERING RESEARCH LOOKING at how nanoscale properties, such as nano-patterning and elasticity, impact on human cells has the potential to create sophisticated blueprints for regenerative medicine. Some of this innovative work underway at the Department of Electrical and Computer Engineering at Michigan State University, USA, delves into how the nanophysical properties of tissue scaffolds can provide important directive cues for cell regeneration. The Principal Investigator for Nanoscale Cues for Regenerative Neural Cell Systems, Professor Virginia Ayres, is exploring the assumption that a set of nanoscale physical and biochemical properties provide directive cues to re-establishing cell systems.

For a number of years, tissue scaffolds have been employed in regenerative medicine. The tissue scaffolds are actually synthetic versions of local extracelluar matrices (ECMs) that all cell types, except freely floating blood cells, need to thrive. Synthetic ECMs can be fabricated from electrospun nanofibres, porous hydrogels, or allografts of cleansed and stripped native ECM. A synthetic tissue scaffold can be designed to slowly biodegrade; it disappears over time as the healthy cell system begins to manufacture and maintain its own local ECM.

To enhance the therapeutic possibilities of synthetic ECMs, Ayres and her team are investigating the nanoscale properties of the nanofibrillar scaffolds: "It is only recently that the physical properties of the scaffold itself have come under investigation as potentially directive," she explains. The researchers aim to utilise this novel knowledge-base for further study opportunities in regenerative medicine, specifically for the central nervous system (CNS: brain and spinal cord). The researchers have an interest in a particular nanofibrillar scaffold environment, composed of electrospun polyamide nanofibres. Some promise for CNS repair, both in *in vivo* and *in vitro* situations, has already been demonstrated.

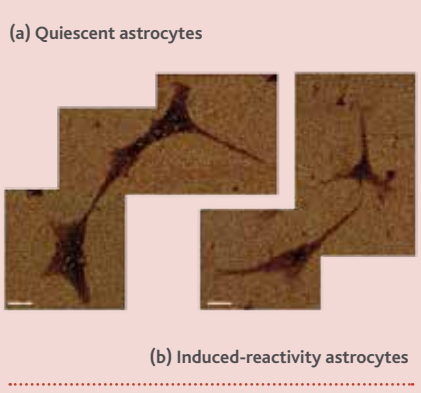

FIGURE 1. Quiescent astrocytes **(a)** and induced-reactivity astrocytes **(b)** cultured on nanofibrillar scaffolds demonstrate almost unchanged morphologies. These are AFM Gaussian high pass filtered composite height images. The scale bars shown are 20 µm.

BUILDING ON SUCCESS

Collaborative experiments that supported the early stages of this research have gleaned some exciting results. During *in vivo* investigations, electrospun polyamide nanofibrillar scaffolds were introduced into spinal cord wound sites of rat models and found to promote accelerated hind limb recovery. Results were measured using a standardised observational scoring; and in response to the extracellular scaffolds, functional neurons and functional blood vessels were observed returning throughout the wound sites.

In addition, at three and five weeks following injury, lower levels of astrocytic scarring (in comparison to controls) were recorded. Astrocytic scarring is a major cause of paralysis, it biomechanically and biochemically blocks the reconnection of neurons and axons across a wound site. The *in vitro* research highlighted that astrocytes cultured on nanofibrillar scaffolds assumed morphologies that appeared to replicate those observed in native tissues. Furthermore, an increase in neurite outgrowth by co-cultured neurons was demonstrated.

Overall, the therapeutic effect of introducing these nanofibrillar scafffolds with their particular set of properties has potential applications in tissue injury repair: "The introduction of these nanofibrillar scaffolds therefore appeared to trigger a number of beneficial responses," expounds Ayres. The focus of the group's work became to determine just why, and how, this is happening at the fundamental level – the nanoscale.

AN INTEGRATED PHYSICS-CELL BIOLOGY EFFORT

The first step required isolating properties of the scaffolds that were potentially directive for tissue growth. The researchers employed a range of innovative nano-investigative techniques, including scanning probe recognition microscopy (microscopy techniques developed by Ayres and her team) to assess the surface roughness, curvature and local elasticity along nanofibres; transmission electron microscopy to investigate nanofibre internal structure(s); micro raman spectroscopy to analyse chemical composition; and contact angle analysis to measure surface polarity.

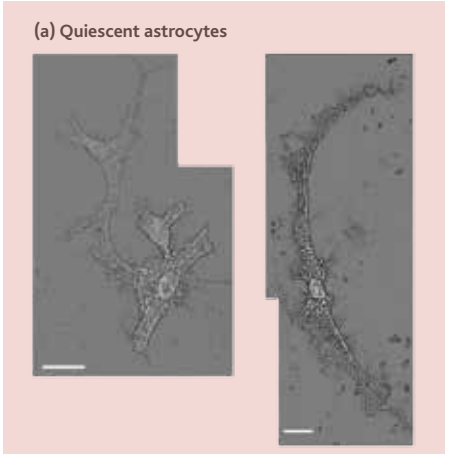

FIGURE 2. Quiescent astrocytes **(a)** and induced-reactivity astrocytes **(b)** cultured on poly-L-lysine functionalised glass, a common cell culture environment, demonstrate greatly changed morphologies. These are AFM composite deflection images. All scale bars shown are 20 µm.

The next stage was to analyse the reaction of the CNS neural cell system to the nanofibrillar scaffolds vis-à-vis conventional cell culture substrates – targeting the astrocyte response. "The *in vitro* studies indicated that the nanofibrillar scaffolds modulate the astrocyte response, which in turn favourably impacts the neurite outgrowth of co-cultured neurons," elucidates Ayres. "That's doubly interesting, because it is astrocytes on the warpath (reactive astrocytes) that create the paralysis-causing glial scar in an injury situation." Distinguishing between cellular edges and the background nanofibres during cell morphology investigations required the team to develop additional and advanced methods of utilising atomic force microscopy (AFM) in combination with image processing. A special image filter was designed so that the researchers could clearly segment the cells from the nanofibres in the AFM images. The greater feature resolution provided valuable information in support of their hypothesis, as Ayres notes: "This new imaging revealed that the nanofibrillar scaffolds enabled the establishment of previously unrecognised astrocyte cell-cell interactions". (See figure 1).

NANOSCALE CUES FOR REGENERATIVE NEURAL CELL SYSTEMS

OBJECTIVES

To investigate the hypothesis that a fundamental set of nanoscale physical and biochemical properties serve as the primary cues for the re-establishment of cell systems that rely on nanofibrillar scaffolds in their native environment.

KEY COLLABORATORS

Professor David I Shreiber; **Dr Ijaz Ahmed**, Department of Biomedical Engineering, Rutgers, The State University of New Jersey, USA

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VIRGINIA AYRES earned her PhD and MSc in Physics from Purdue University and two BAs in Physics and Biophysics from Johns Hopkins University. Ayres is currently Associate Professor in the Department of Electrical and Computer Engineering at Michigan State University. Her research interests are in nanobiology, nanoelectronics and scanning probe microscope instrumentation development.

Nanoscale Cues for Regenerative Neural Cell Systems is supported by the National Science Foundation (NSF) Physics of Living Systems Program (Dr Kraston Blagoev, Program Director). Previous NSF awards have supported the development of scanning probe recognition microscopy, used in the present research. Ayres is also the recipient of two NASA Faculty Fellowship Awards for nanoelectronics research at the NASA Goddard Space Flight Centre, and two international awards for research and education from the Japan Society for Promotion of Science and as Chair of International Cooperation Visiting Professor at Tokyo Institute of Technology.

Ayres has been honoured to serve as visiting Program Director (Rotator) in the NSF Engineering Division and continues to serve the scientific community as a peer reviewer for NSF and for the National Institutes of Health (NIH).

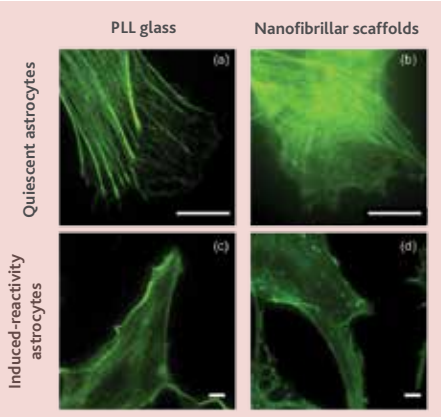

FIGURE 3. Quiescent astrocytes cultured on poly-L-lysine (PLL) functionalised glass **(a)** have stress fibres for cell motility. On the nanofibrillar scaffolds **(b)**, the stress fibres are dis-assembled as part of a stellation response induced solely by the nanofibrillar environment. In dBcAMP-stimulated astrocytes, a stellation response is chemically induced with similar results for cells cultured on PLL glass **(c)** and nanofibrillar scaffolds **(d)**. Note the similarity of the induced-reactivity images **(c)** and **(d)** to the quiescent nanofibrillar scaffold image **(b)**. These are F-actin staining fluorescence images. The scale bars shown are all 20 µm.

EXPLORING ASTROCYTE RESPONSES

During 2012, a novel combined investigation of the morphological and signalling protein responses to a subset of the nanoscale properties was undertaken for quiescent (healthy, non-reactive) astrocytes. Proteins that are part of a signalling pathway directing cells to change shape were targeted (see figure 2). A key component of this included exploiting 3D (z-series) laser scanning confocal microscopy to quantitatively measure the protein expressions within the cells, with a focus on the upstream regulators of filopodia, lamellipodia and stress fibre formation. The most important feature of healthy astrocytes, literally 'star cells', is stellation, which happens when the stress fibre response is turned off (see figure 3). A unique stellation response was seen only for the astrocytes cultured on nanofibrillar scaffolds, and it included depression of the stress-fibre inducing protein. "As only external cues were used to trigger the responses, the results thus supported the hypothesis that cues from the extracellular environment (such as by the nanofibrillar scaffolds) can trigger preferential activation of members of the Rho GTPase family with demonstrable morphological consequences for cerebral cortical astrocytes," observes Ayres.

Further investigation, completed during 2013, looked more closely at the ways in which the nanofibrillar scaffold cues help to reduce and mitigate astrocyte reactivity that results in the neuron blocking glial scar formation. This was done by repeating earlier experiments, but this time using cerebral cortical astrocytes which had been purposely set to be reactive. The astrocytes on nanofibrillar scaffolds reponse was again unique in that it was, to within statistical error, unchanged. And the reactive astrocyte study yielded a second important finding: that a different set of nanoscale property values from one of the comparison culture environments induced the responses of glial scar formation. These include astrocytes in chain-like clusters with intertwined processes (see figure 4). The morphological and protein expression response differences and similarities were more consistent with those seen in the elasticity property. "This indicates that the nanophysical cues of the extracellular environment could also modulate the reactive astrocyte response but that different cues could have more directive importance for astrocytes in scarring mode versus in non-scarring quiescent mode," Ayres concludes.

This innovative research is reliant upon the active participation of students and collaborators alike. Ayres describes it as a "fusion of physics, biophysics, bio-engineering, electrical engineering and clinical medicine". There is plenty of multidisciplinary scope for students to delve into individual interests and become part of a wider network of investigations and expertise. Whilst working out precisely how the nanoscale cues are perceived, and by which receptors still remains to be established, it is hoped that the findings to date will act as a prototype for developing regenerative treatments, in particular for those which are focused on recreating the neural cell system following CNS injury. In addition, Ayres implicates that the studies have far-reaching potential for exploring regenerative cell-scaffold systems throughout the human body – by identifying each fundamental set of nanoscale properties for supporting local cell system physiology and function.

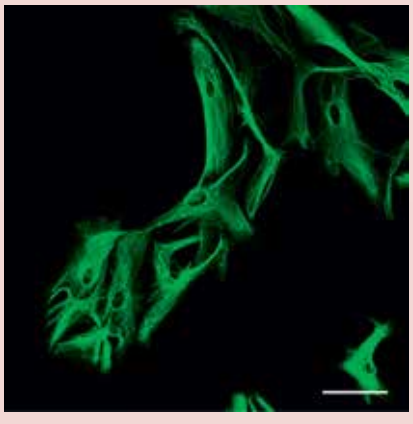

FIGURE 4. Induced-reactivity astrocytes cultured on poly-L-lysine functionalised Aclar. Maximum intensity projection glial fibrillary acidic protein (GFAP) staining image shows the chain-like clustering and intertwined processes associated with glial scar formation. Scale bar, 50 µm.