PERSPECTIVE

"To measure is to know": how advances in image analysis are supporting neural repair strategies

Neuronal regeneration in the peripheral nervous system arises *via* a synergistic interplay of neurotrophic factors, integrins, cytoskeletal proteins, mechanical cues, cytokines, stem cells, glial cells and astrocytes. A plethora of additional potential factors are yet to be discovered (Yiu and He, 2006). In spite of encouraging progress, axonal repair in the central nervous system (CNS) remains elusive. There is a growing consensus that in order to capture such a moving target, a systematic approach has to eclipse the "trial and error" methodology that has proven so effective in many other instances. The role of large scale automation is critical in such an undertaking. This philosophy has in fact been championed for many years already by Big-Pharma together with larger academic laboratories under the heading: "High Content Analysis" (HCA). Neuronal cells in 2D microplate cultures are generally used (see **Figure 1A**). They are exposed to libraries of test compounds and variable environmental conditions as their response is being measured (*e.g.*, axonal length, nucleus size, number of branching points…). More complex model systems are gaining traction, including cell co-cultures, neural explants, 3D cell cultures, organotypic slices, or whole organisms such as zebrafish. But traditional neuronal cell cultures remain a workhorse of research against neurodegenerative diseases.

Figure 1A demonstrates the typical: "native" morphology of neuronal cells in culture stained using biological markers. We note that the image shown here was acquired under fluorescence microscopy, but similar images are produced using many other contrast mechanisms and microscope types. This includes differential interference contrast microscopy or electron microscopy. Our image analysis methods are broadly applicable to neuronal images, although in some instances, computerized pre-processing may be required. In **Figure 1B**, the cell body is outlined in green and neurites have been colored according to branching levels (primary neurites connected to mother cell body, secondary neurites connected to primary neurites…). The process of going from a microscopy image of cells to a set of discrete regions identifying the location of functional cellular subunits is referred to as "image segmentation". This type of image analysis is the bridge beyond a merely qualitative "trial and error" gauging of individual images. It allows reducing the formidable combinatorial complexity inherent in neuronal images to a few numerical parameters capturing functionally relevant aspects. Biological discovery then acquires the systematic character that allows mining for information, testing statistical hypotheses, and guiding experimental strategies in an informed manner. These new powers given to those who exploit image analysis software in their research however, may lead to complacency. We contend that accelerated progress in the neuro-regeneration of the CNS will depend at least in part, on the realization that the "knowledge" gained from image analysis results is only as useful as their quality allows. Alternatively, to make our point more explicit: "Garbage in, garbage out". Such an unforgiving attitude, when applied to **Figure 1**, would allow one to realize that our results may still be improved, a point to which we will come back to later. The critical step upon which the whole HCA approach depends, does not reside in the creation of colorful scatterplots produced by visualization software; it rests mainly on the attentive examination of the image analysis results that must imperatively be confronted to the original image data. By juxtaposing representative samples of the image analysis results onto the original images in an alternating manner (*e.g.*, overlay the results for one second, no overlay for 1 second, overlay results for 1 second …), one not only gains an appreciation of the quality of the results, but can also improve them incrementally by exploring interactively the effect of parameter variations. This capability has been a central feature of our own solution to neuronal image analysis since its inception (www.hca-vision.com (Vallotton et al., 2007; Wang et al., 2010)). Yet we do not see this principle adopted universally: too often, the user still need to confront a panel containing dozens of parameters and the usual way forward is to adopt parameters from the last user – rather than those that would be best suited to a new image data set. We see several possible reasons for this state of affair: One is that very fast image processing must be achieved to make the approach practical. Our own research has been underpinned by the use of fast algorithms, parallel computing, and GPU computing (Sun and Vallotton, 2009; Domanski et al., 2011). We anticipate that real-time image processing still has much more in store for improving the drug discovery and development pipelines of the future.

Whereas speed is inherently important, the ability to precisely delineate the paths of neuronal projections even in very weakly contrasted images is another area that needs further progress. In previous work (Vallotton et al., 2011), we introduced a new image transform (the "coalescing shortest path transform") which showed promise in highlighting very weak linear features in a pre-processing step. With ever increasing processing power and new hardware architectures, this new image transform will find more widespread adoption in spite of its significant computational complexity.

The results of the image segmentation must often be further processed and tailored to the biological question at hand. In one experimental approach, Carbon nanotubes were arrayed parallel to one another on a gold mylar surface, and the ability of the functionalized surface to guide axonal growth anisotropically is to be measured and optimized (**Figure 1**). In principle, image

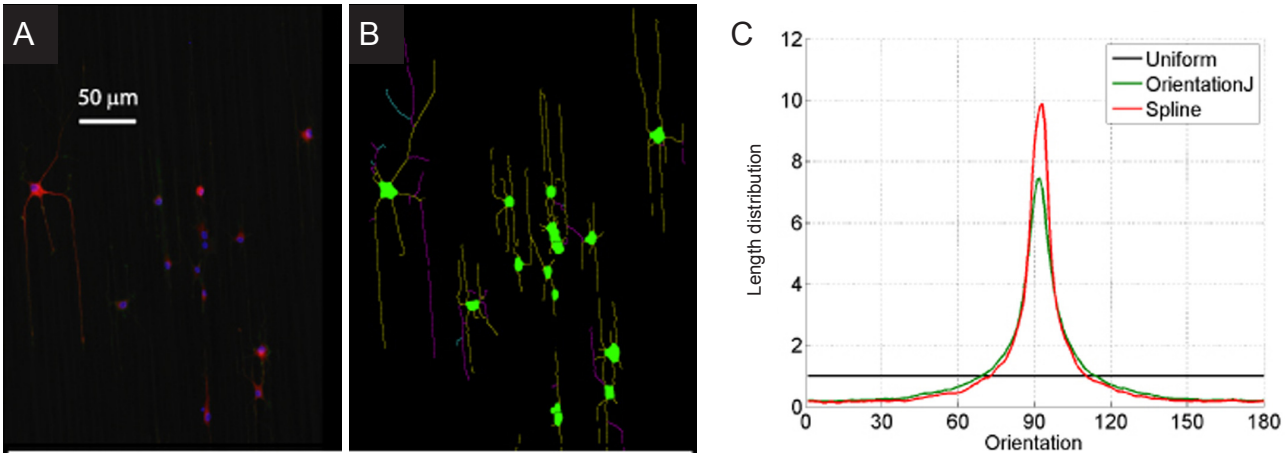

Figure 1 Characterising axonal growth anisotropy using automated image analysis.
(A) Representative image of neuronal cells stained with βIII tubulin (red) to provide neuronal structure visualisation and 4′,6-diamidino-2-phenylindole (blue) to provide referential data for individual cells. (B) Result of image segmentation. Dilated cell bodies are displayed in green and neurites are coded according to branching levels. Even faint neuronal projections are detected and traced, in spite of a rather complex background. Arrays of parallel carbon nanotubes on the surface (which are responsible for the structured background signal in A) confer a highly anisotropic character to the growth of neuronal projections in the vertical direction. (C) We have developed dedicated tools to measure this anisotropy precisely using orientation histograms - see Payne et al., 2014 for details.

anisotropy could be measured on an average basis, but what is really of interest in this case is the alignment of the neurites themselves with the nanotubes – the rest of the image being largely irrelevant. We have thus built on our ability to measure the paths of neurites reliably in order to restrict the measurement of anisotropy to the relevant neurites. Using this methodology, a spectacular increase of neurite guidance in the direction of the carbon nanotubes was demonstrated in a reliable quantitative manner (Payne et al., 2014). This methodology will soon be applied to evaluate the anisotropic effects of the nanostructural guidance cues combined with an applied electrical potential.

On a digital grid, the local neurite traces may only point in 8 cardinal directions (as there are 8 pixels directly in contact with an arbitrary image pixel). This angular resolution is far too poor to measure subtle anisotropy effects induced by external cues. We thus introduced smoothing splines (continuous curves that represent an optimal fit to the discrete traces in the image raster) in order to capture neurite orientation information with high precision. Based on these analytical curves, it is an easy matter to generate orientation distributions that quantitatively capture the propensity of neurites to align along a favored direction (**Figure 1C**). Significant efforts has been expended on the correct sampling of angular orientations along neurites and our code is thus available as supplementary information to Payne et al. (2014).

Additional features of neuronal projections are equally relevant from a regeneration point of view. For example their width relates to the tendency to grow and develop into axons, or instead, to retract and sometimes degenerate. To cater for this, we developed an image analysis approach based on the use of geodesics (shortest paths) in derivative images, enabling detailed measurements of neuronal widths (Lagerstrom et al., 2008). This type of technology plays an important role in improving understanding of the dynamics of neuronal outgrowth. It may also be used for early identification of axonal protrusion amongst all other protrusions, on the basis that axons tend to be thickest among neurites.

More familiar measurements of neuronal trees, such as the average degree of branching or the length of the longest projection are also relevant to research in neural regeneration. However, as they are now almost universally available in software solutions from original equipment manufacturers, we do not elaborate on these further.

Automated image analysis tools have significantly progressed in recent years but much remains to be achieved: For example, the analysis of dense neuronal cultures remains a challenge. In this situation, correct attribution of neuronal projections to their true mother cell has not received sufficient attention. In addition, the segmentation of growth cones is rarely addressed correctly (if at all), and dealing reliably with crossing neurites still remains aspirational. In principle, these are not insurmountable problems, but progress will inevitably be gradual. To a certain extent, the relative lack of advance in these areas stems from the perception of many users (and developers) that image analysis tools are already adequate as they stand. Indeed, it can be anticipated that most of the sigmoidal dose response curves produced in a "Big Pharma" setting will not shift spectacularly when the problems mentioned above are finally solved. Thus, progress is more likely to be predicated and achieved by academic research collaborations whose particular objectives critically depend on superior image analysis quality. The competitions that are occasionally organized

by commercial entities or by dedicated conference organizers will tremendously benefit the field too. Catalytic progress will also likely arise from entirely different application areas, such as retinal blood vessel tracing, which is increasingly used as a biometric. In this type of applications, tracing mistakes - even rare - may have dire consequences, such as denial of access, or even judiciary blunders.

Finally, we predict that considerable improvements will also come from dynamic imaging. In this case, live neurons rather than fixed ones are examined and ambiguous results stemming from limited observation times can be corrected by the consistent comparison of segmentation results across multiple frames. Coincidentally, live neuronal imaging is gaining prominence in drug discovery and development because the method provides a wealth of information not available from endpoint assays. This type of dynamic imaging, particularly as far as the tracking of compact objects through time-lapse sequences is concerned has made considerable progress over the last few years. We have ourselves been involved in the development of a powerful framework for object tracking that has been successfully applied to many problems, such as the transport of viruses along axons, or cell tracking (www.diatrack.org (Vallotton and Olivier, 2013)). By combining technologies developed in this latter context with the body of technologies underlying the analysis of neuronal populations, we feel that transformational progress is a near.

Time will tell if our ability to weave increasingly complex nanotubes patterns in order to stimulate and guide neuronal growth will represent an effective approach to CNS and/or PNS repair in a clinical setting. But independently of this important question, automated image analysis will remain a central tool for a rapidly expanding area of research.

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