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Application of computer-assisted image analysis for identifying and quantifying liver fibrosis in an experimental model

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ABSTRACT

Liver fibrosis and resultant cirrhosis are amongst the most common outcomes of chronic liver diseases. Currently, liver transplantation remains the only effective treatment. Thus, a reliable and objective method is fundamental for quantifying fibrosis adequately, which is essential to prognosis, diagnosis and evaluation of response to antifibrotic therapies. Visual analysis of biological samples using semiquantitative scoring systems have been described, but all are time-consuming, qualitative, and produce partially subjective fibrosis evaluations with only moderate accuracy. While numerous commercial software packages exist for image analysis, many of these packages are designed for very specific purposes, are expensive and proprietary, meaning that the underlying methods of analysis are hidden from the researcher. On the other hand, the design of the free software ImageJ/National Institutes of Health (NIH) Image is geared more toward the analysis of individual images (comparable to Adobe Photoshop) rather than flexible, high-throughput work. Toward the aim of identifying alternative analytical approaches for precise quantification of a large number of histological images of liver fibrosis, this paper describes the configuration and use of an open-source automated image analysis software, CellProfiler, for quantification of fibrosis induced in rats. Once fibrosis had been established, liver samples were collected, histologically processed and subjected to CellProfiler image analysis, which automatically identifies and isolates fibrosis according to staining, and then measures the area occupied by fibrosis over the total liver area examined. CellProfiler was shown to be an objective, precise and rapid method that allowed simultaneous quantification of fibrosis in all six hundred histological images of injured liver examined, at a rate of ~ 10 s/image. This novel tool might be of special value to allow the drawing of valid conclusions regarding the applicability of regenerative therapies to treat liver fibrosis in experimental studies, and also opens the way for further investigations aimed the extending the use of CellProfiler to other tissue assays.

Keywords: computational biology, automated image analysis, image processing, CellProfiler software, liver fibrosis.

1 INTRODUCTION

Liver fibrosis and resultant cirrhosis are amongst the most common outcomes of chronic liver diseases following diverse types of insults, such as viral infection, alcoholism or chemical toxicity. It is often associated with severe morbidity and significant mortality, eventually resulting in the need for liver transplantation [1, 2]. Thus, in both clinical practice and *in vivo* experimental models, it is fundamental to have a reliable and objective method for precise quantification of liver fibrosis, as this is essential for prognosis, diagnosis and evaluation of response to antifibrotic therapies. Histologically, fibrosis is characterized by an excessive accumu-

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lation of collagen (or fibrous tissue) in the liver parenchyma that distorts the normal hepatic architecture, with the formation of scar tissue encapsulating the area of injury, therefore leading to impairment of liver function [3].

A routinely used method for liver fibrosis assessment is microscopic evaluation of the trichrome-stained liver biopsy specimen. Traditionally, the method for fibrosis assessment in liver biopsies involved grading of the fibrosis as absent, mild, moderate, or severe. In the 1980s, semiquantitative scoring systems emerged as an alternative to this simple approach, and these have since been used to evaluate liver fibrosis [4, 5, 6]. These systems are based mainly on classification of fibrosis into stages according its localization and the pathological pattern of the hepatic structure, providing an indication of the relative severity and of disease progression. However, in spite of attempts to improve the characterization and objectivity of these methods by introducing new scores [7], some limitations remain. Firstly, while these systems remain valuable for describing the histological extent of liver fibrosis, their precision is moderate at best, whilst at intermediate stages of disease, they are subjectively dependent on the visual interpretation of the observer, who must be an experienced pathologist [8]. Secondly, studies aimed at validating some of these systems have shown varying degrees of intraobserver and interobserver reproducibility [9, 10], whilst the semiquantitative scores obtained are not sensitive enough to detect small changes in fibrosis [11]. Thirdly, although visual analysis is quite a powerful method for examining small samples, observation of many samples by eye is time-consuming, subjective and nonquantitative [12].

Toward the goal of overcoming the majority of these problems, several studies have used digital analysis quantification methods [8, 13, 14, 15]. For example, computerized image analysis has several advantages over visual analysis using semiquantitative scoring systems, including reproducibility, rapidity, adaptability and the ability to simultaneously measure many features in the image. Moreover, automated quantification techniques offer greater statistical power in the analysis of data obtained [8]. Numerous software programs are used daily in laboratories to analyze biological images; examples include Image-Pro Plus (MidiaCibernetics, www.mediacy.com); and the MetaMorph® Imaging System (Universal Imaging Corporation, www.universalimaging.com). Some packages are designed for a very specific purpose, such as cell counting [17], while others are sold with accompanying hardware for image acquisition (e.g., yeast colony counters). Unfortunately, these software programs are expensive and do not allow measurement of features beyond those that are already built-in. In addition, most commercial software is proprietary, meaning that the underlying methods of analysis are hidden, therefore preventing researchers from understanding the strategy of a given algorithm or modifying it to suit their particular requirements [12, 18]. On the other hand, while the free software package Image J / National Institute of Health (NIH) Image [19] has been used by some laboratories, its design is geared more toward the analysis of individual images (comparable to Adobe Photoshop) rather than flexible, high-throughput work. According to Carpenter et al. [18], macros can be written in Image J for high-throughput work, however adapting macros to new projects would require biologists to learn a programming language. In addition, most of the custom programs written in commercial or free software are not modular, and therefore, the combining of several steps and changing of settings requires direct manipulation of the code, and is simply not practical for the processing of hundreds of thousands of images.

In seeking alternative analytical approaches which would allow precise quantification of a large number of histological images from fibrotic livers, this paper describes the configuration and use of an open-source automated image analysis software, CellProfiler, for quantification of fibrosis induced in rats by the bile duct ligation procedure.

2 OVERVIEW OF THE SOFTWARE SYSTEM

CellProfiler is an open-source software tool for quantifying data from biological images, particularly in high-throughput experiments, with functions including measurement of size, shape, intensity and texture of every cell, cell cycle distribution and various other features of individual cells or tissues in images. The compiled software is freely available for Macintosh®, PC and Unix plataforms at www.cellprofiler.org. Using the point-and-click graphical user interface (GUI), users construct an image analysis "pipeline", that is, a sequential series of modules that each performs an image processing function such as image processing, object identification (segmentation), and object measurement. The measurements can be viewed by (a) using CellProfiler's built-in viewing and plotting data tools; (b) exporting into a tab-delimited spreadsheet format that can be opened in programs such as Microsoft Excel and OpenOffice Calc; (c) exporting into a format that can be imported into a database such as Oracle or MySQL; or (d) directly in MATLAB. Over 50 CellProfiler modules are currently available. Most modules are automatic, but the software also allows interactive modules (for example, the user can click to outline a region of interest in each image). Users can

also mix and match modules and adjust their settings in order to measure the object of interest. While originally designed for high-throughput images, the software is equally appropriate for low- throughput assays (i.e., assays of < 100 images). [12, 18].

The website www.cellprofiler.org offers the possibility of downloading videos and written tutorials showing CellProfiler's features and instructions for their use. Many example pipelines are also provided at the CellProfiler website in order to provide a starting point for new analyses, while an online discussion forum has also been established to allow sharing of information regarding the software. In addition, the modular design and point-and-click interface make setting up an analysis feasible even for those without a programming background. When performing the same type of analysis on different image sets where sample preparation is the only variable, the analysis is first tested on a few sample images, which allows the changing, if necessary, of some of the parameters in the Identify modules. Once a pipeline is deemed to be satisfactory, analysis can either be performed on the local computer, or automatically divided into smaller batches to be sent to a cluster of computers. CellProfiler's code is open-source under the GNU public license, and its image handling is flexible in that there is no requirement for images to have a certain naming structure, and many standard image formats are supported. The modular structure of the software also allows experts to expand the software to new file formats or add new algorithms [12, 18].

3 METHODOLOGY

3.1 Animal model of liver fibrosis

All animal experiments were carried out in accordance with current Italian and European regulations and laws on the Use and Care of Animals for research (DL. 116/27 January 1992). Thirty female Wistar rats (Laboratoire Elevage Janvier, France), weighing 220-250g, were submitted to Bile Duct Ligation (BDL), a classical model for inducing liver fibrosis [20]. BDL is a surgical procedure which involves the insertion of double ligatures with suture silk in the common bile duct, with a transaction then performed between the two ligation points. This model causes permanent biliary obstruction, which introduces biomechanical stress to the liver tissue and triggers an inflammatory process, which ultimately leads to fibrosis. According to Li and Crawford [21], the BDL model closely mimics the fibrotic pathology that arises in a variety of human liver diseases, such as congenital biliary atresia, biliary cirrhosis, or diseases occurring as a consequence of gallstones or compression of the bile duct by tumors.

3.2 Tissue sample preparation

Two, 4 and 6 weeks after the induction of fibrosis, ten animals at each time point were sacrificed to allow collection of liver for histological processing. The liver tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm thickness and stained with Goldner's Masson Trichrome for digital image analysis. Goldner's Masson Trichrome stains fibrous tissue green and parenchymal cells red. To avoid potential variability in the staining intensity of histological sections from different staining batches, all sections were stained using the same Goldner's Masson stain kit.

3.3 Tissue sample digitalization and image analysis

Six hundred colored histological images resulting from Goldner's Masson Trichrome staining were captured by a digital camera (Olympus Camedia C-4040 ZOOM) and digitalized at 1024×768 pixel, 24 bit/pixel resolution, from 10 non-overlapping random fields per histological section, at a global magnification of $\times 100$. The digital images were processed using the CellProfiler cell image analysis software installed on a PC computer Intel Pentium Core 2 (CPU T7200, 2GH2, 2.50GB HAM). The cellprofiler automatically identifies and isolates fibrotic areas according to their staining (green) and then measures the area occupied by fibrosis with respect to the total liver area examined. The percentage of area occupied by fibrosis (PF) is then determined, according to the following equation, PF $=$ (labeled fibrosis area/total image area) $\times$ 100.

4 SETTING UP AND USING CellProfiler

4.1 Before the image analysis run

Once the CellProfiler software has been installed, a pipeline consisting of a sequential set of image analysis modules is set up. To assist with this, an example pipeline can be loaded and run on the example images provided on the CellProfiler website, to allow the user to observe how processing typically proceeds. The example pipeline can then be adapted to the images of the experiment. Alternatively, a pipeline can be built from scratch, which involves the placing of individual modules into a pipeline. A typical pipeline consists of modules of the following categories: file processing (e.g. LoadImages and SaveImage), image processing, object identification, and measurement. Upon commencement of the analysis, each image (or group of images) travels through the pipeline and is processed by each module in order. The diagram in Figure 1 shows a general overview of the CellProfiler pipeline which is used for fibrosis analysis.

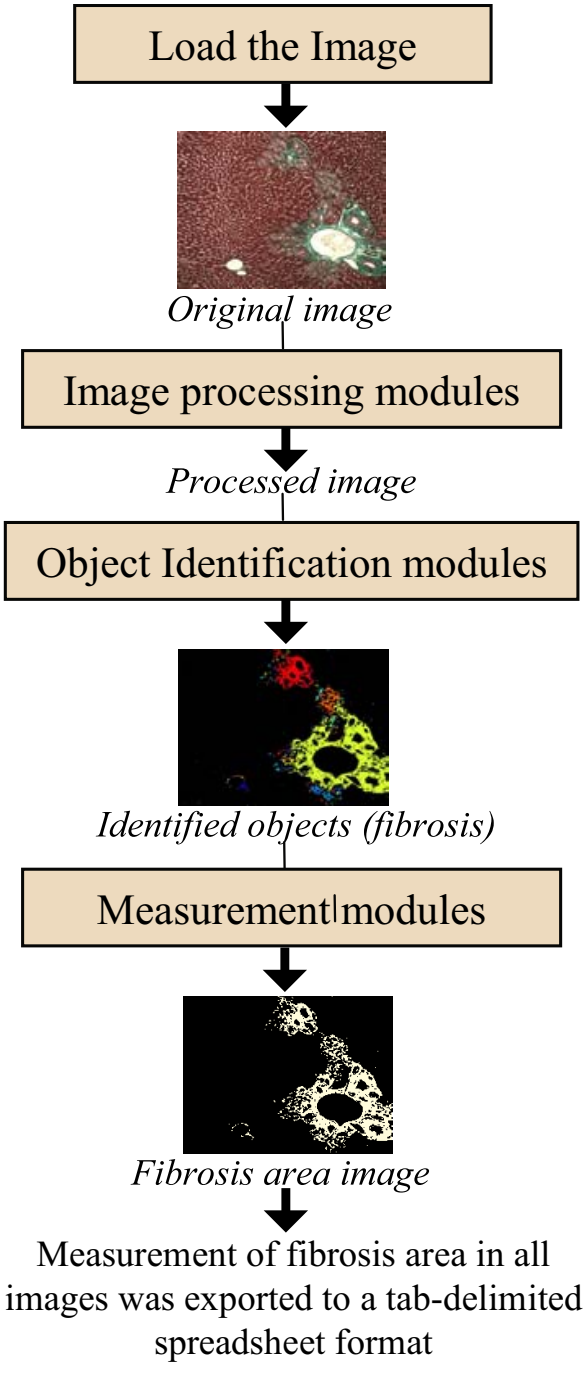

Figure 1 – Schematic representation of a typical CellProfiler pipeline. The modules are placed in sequential order to create a pipeline which usually consists of loading the images, processing the images, identifying the objects, and taking measurements of those objects. The names of the images created appear in italics below each image, whereas the module names appear in a larger regular font.

Using the CellProfiler window or main CellProfiler interface (Fig. 2), modules can be added, removed, or rearranged within a pipeline by clicking the *Add, Remove* or *Move up* and *Move down* tabs which are located underneath the pipeline panel. By clicking on a module in the pipeline panel, its settings will appear in the main workspace where they can be adjusted according to the image that is being analyzed. Next, the *Default Image (Input) Folder, Default Output Folder* and *output filename* must be set up, using the *Browse* tab and the computer's normal interface. In the Folder panel, the *Default Image Folder* contains the input image (or a group of images) which is to be analyzed, while the *Default Output Folder* is used by CellProfiler to store the output files which it creates, and contains the processed images (i.e. resulting images).

The first module is the LoadImages module, which allows the user to specify which images are to be loaded and in which order. This module also tells CellProfiler where to retrieve images from, and gives each image a meaningful name by which other modules can access it. There are a number of ways whereby images can be loaded and identified. One of these is by selection of *Text-Exact match* in the first text box in the workspace of the module (Fig. 2, arrow). This option is used to load images which have a particular piece of text in their filename, for example, when all histological images contain "tif" in their filenames. This text can be entered in the second text box (Fig. 2, arrow), or alternatively, typing the exact file name of the image will instruct CellProfiler to analyze only the image that has been specified.

4.2 Commencing image analysis

Upon commencement of the analysis, each image that has been loaded travels through the pipeline and is processed by each module according to the pre-adjusted settings, in the sequential order of the pipeline. A window is opened for each module, and in the event that there is more than one image to be processed, the module windows will refresh upon completion of each cycle of CellProfiler processing. The steps of the automated fibrosis image analysis and the resulting images of each step are shown in Figures 3, 4, 5, and 6.

The second module of the image analysis is the ColorToGray (Fig. 3). This module splits the *Original Color* image into three separate images: red, blue, and green. Each of these images is then converted to an image with varying intensities on a grayscale, and these are then used for separate purposes later in the pipeline. For example, in our case the *Red* image was chosen as the input image for the next module. This can be decided based on a visual inspection of which images (red, blue, or green) show the best contrast for fibrosis compared to background, or by using CellProfiler Image Tools > ShowOrHidePixelData to check the contrast in each channel numerically. The

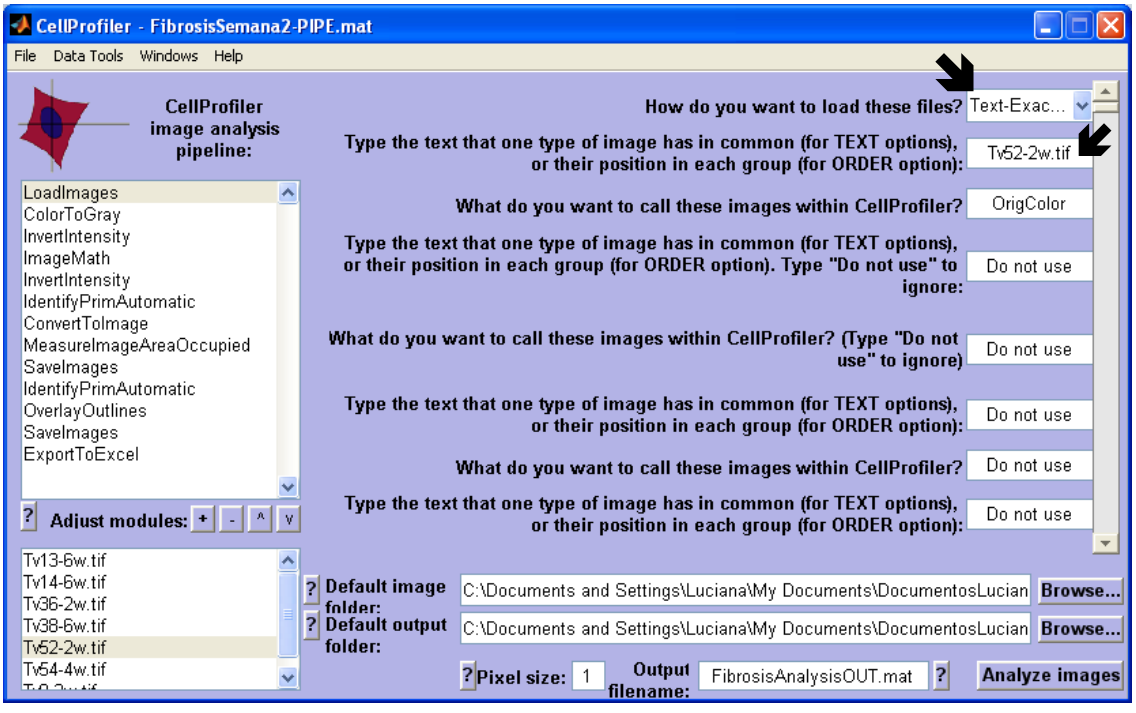

Figure 2 – Main CellProfiler interface with the pipeline for fibrosis analysis displayed. Pipeline panel and the tabs for adjusting the modules **(upper left of the window)** ; the workspace with the settings of the LoadImages module (**upper right of the window**); the text box to specify the way images are loaded and identified (↘); the text box with the exact file name of the image to be analyzed (↙); the folder panel used to set up the *Default Image Folder*, *Default Output Folder* and the *output filename* (**lower right of the window**); the file panel, where the contents of the *Default Image Folder* are shown (**lower left of the window**).

ColorToGray module is especially helpful because the Identify module requires grayscale images. Next, the intensity of the grayscale *Red* image is inverted by the InvertIntensity module, so that black becomes white and *vice versa*. The resulting image of this module is called an *InvertedRed* image (Fig. 4A). The next module, ImageMath, performs a mathematical operation on image intensities in order to enhance the intensity or the contrast of the image. In this step of our analysis, two images were used, namely, the grayscale *OriginalGreen* and the *InvertedRed* image (Fig. 4B). The resulting image of the mathematical operation performed on those images then became the input image of the next module of CellProfiler.

The module IdentifyPrimAutomatic (Fig. 5) identifies regions in grayscale images containing bright objects on a dark background. In our study, objects mean fibrosis or areas occupied by fibrosis. The first setting of this module which required adjustment was the *Typical diameter of object, in pixel units (min, max)*. The estimate of the size range of the objects is important to allow them to be distinguished from noise in the image. The second setting requiring adjustment was the *Select the thresholding method*. The intensity threshold affects the decision of whether each pixel will be considered foreground (regions of interest) or background. A stringent threshold will result in only bright regions being identified, with tight lines around them, whereas a lenient threshold will include dim regions, with the lines between regions and background being more loose. The threshold is automatically calculated using the method *RobustBackground global*, which is selected in the workspace of the module's window. Automatic threshold calculation offers the advantage of being able to adapt to changes in lighting/staining conditions between images, and is usually more robust/accurate. The *RobustBackground global* method trims the brightest and dimmest 5% of pixel intensities, with the view that the remaining pixels represent a Gaussian of intensity values that are mostly background pixels. The method then calculates the mean and standard deviation of the remaining pixels and calculates the threshold as the mean + 2 times the standard deviation. The word *global* is applied as one threshold is used for the entire image. Another setting of this module is the *Threshold correction factor*, which is included to address the fact that an automatically calculated

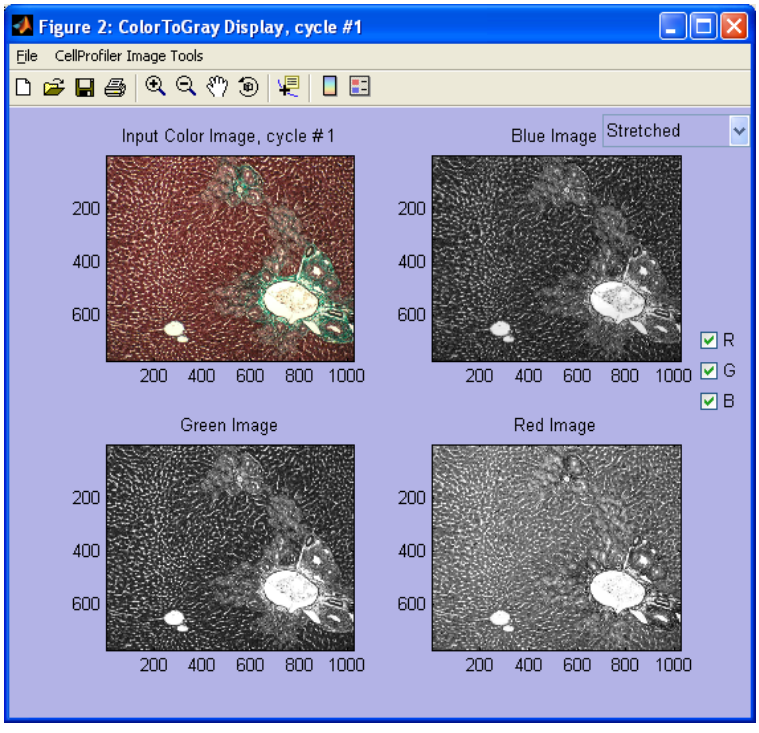

Figure 3 – ColorToGray module: the Input *Color* image is converted into three separate grayscale images (*red*, *blue*, *green* image).

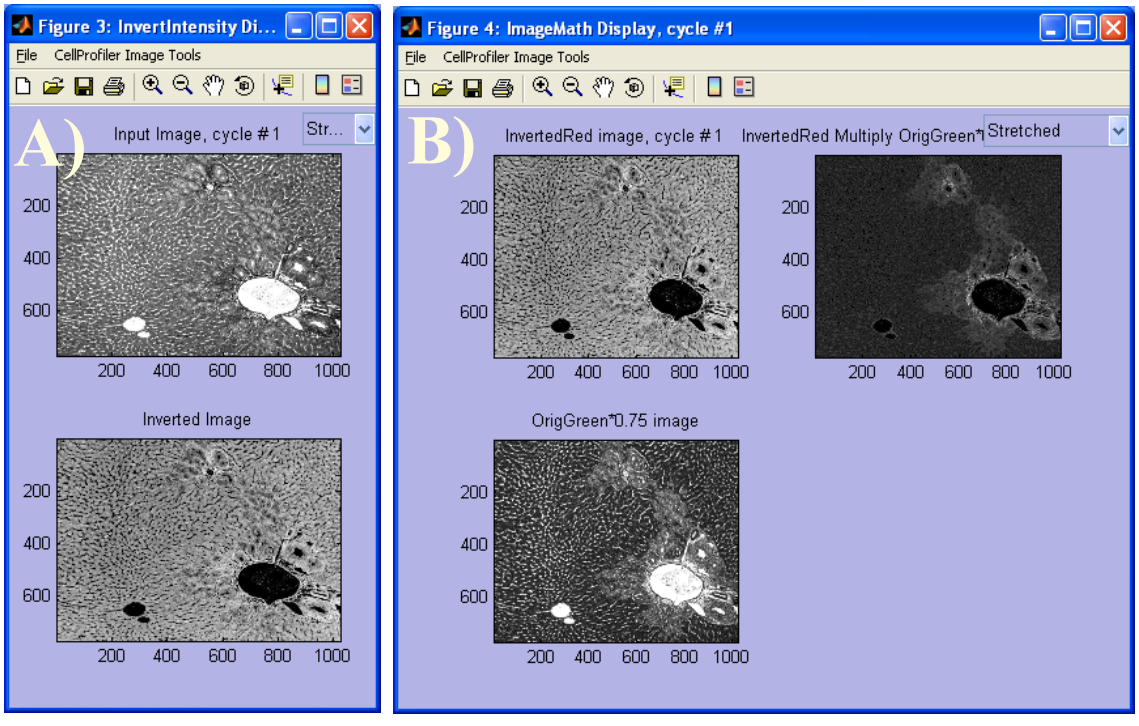

Figure 4 – **A)** InvertIntensity module: the intensity of the Input *Red* image is inverted; **B)** ImageMath module: the grayscale *Green* Image is multiplied by a factor and then by the *InvertedRed* image, resulting in the *ContrastEnhancement* image.

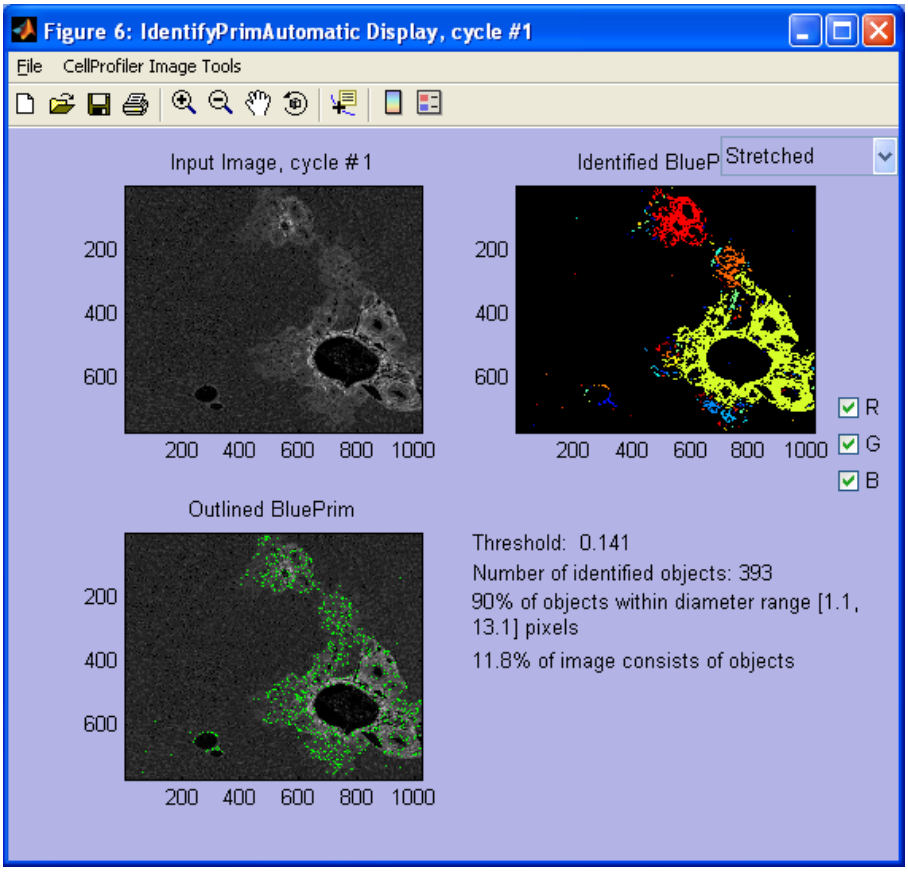

Figure 5 – IdentifyPromAutomatic module: identifies objects (fibrosis) in the input image by selecting the diameter range pixels and applying an automatic threshold method. The *Outlined BluePrim* image shows the fibrosis outlined in green, representing all fibrosis identified. The *Identified BluePrim* image shows the identified fibrosis as arbitrary colors, which help to distinguish the area occupied by fibrosis.

threshold may consistently be too stringent or too lenient, making it necessary to enter an adjustment factor which is empirically determined and suitable for the user's images. A value of 1 means that no adjustment is made and values of 0 to 1 make the threshold more lenient, while a value greater than 1 (e.g. 1.3) makes the threshold more stringent. Figure 5 shows the window for the IdentifyPrimAutomatic method with the input image *ContrastEnhancement* and the two resulting images from this step of fibrosis image analysis, *Identified* and *Outlined BluePrim*.

Once the fibrotic area is identified, CellProfiler automated analysis continues with the ConvertToImage module (Fig. 6A). This module allows previously identified objects to be converted into an image according to a color which has been selected for the resulting image, which can then be saved with the SaveImages module. The color chosen for our analysis was *Binary*, meaning that all object pixels were assigned 1 and all background pixels were assigned 0, creating a binary image.

Finally, the last module in the pipeline is the MeasurmentImageArea (Fig. 6B), which measures the total area occupied by stain in an image and uses a threshold to determine stain and background. This module applies a threshold to the Input Image so that any pixels brighter than the specified value are assigned the value 1 (white) and the remaining pixels are assigned the value zero (black), producing another binary image. Next, the number of white pixels is counted, thereby providing a measurement of the area occupied by the staining. This stained or labeled area represents fibrosis which has already been identified by Identify module. Setting up of this module requires the insertion of an absolute number between 0 and 1 for the threshold. To help in determining the threshold value manually, the pixel intensity is inspected in the image by using the Show intensity histogram, which is available in the Image Tool window that can be assessed by clicking on any image.

Figure 7 shows some of the steps used by CellProfiler to

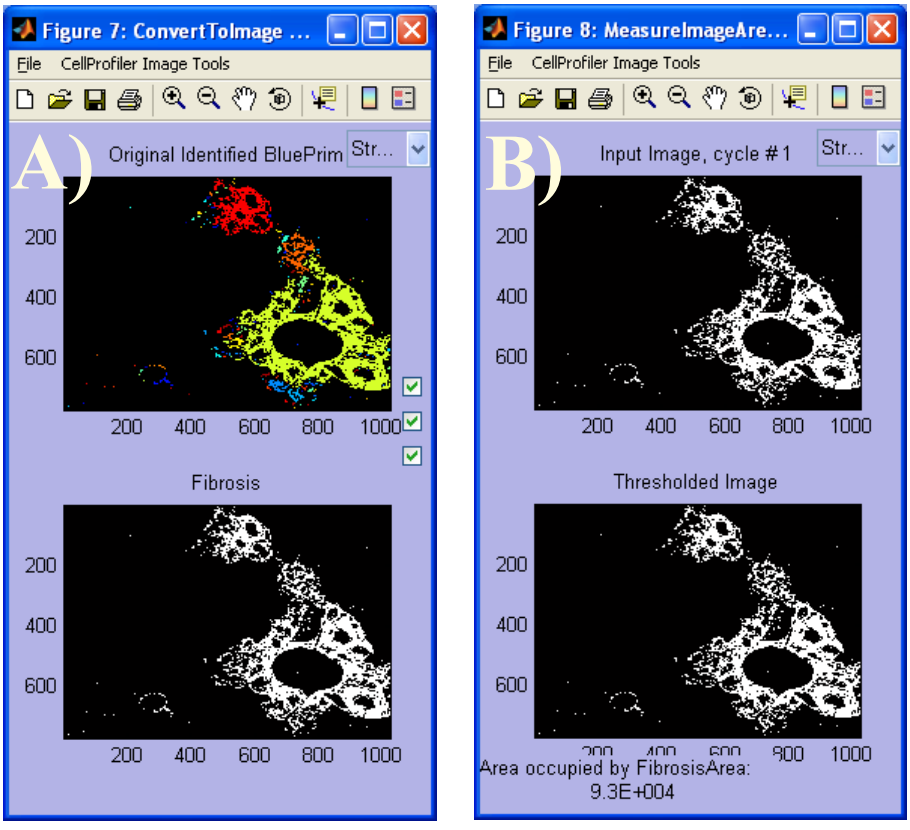

Figure 6 – **A)** ConvertToImage module: the *Original Identified BluePrim* image with the fibrotic area identified is converted into a binary image (black and white), called *Fibrosis*; **B**) MeasureImageArea module: measures the stained area (white) by applying a threshold to the Input Image resulting in *FibrosisArea* image.

automatically isolate, identify and measure the fibrotic area in the overall histological image. The OverlayOutlines module places yellow outlines on the *Original Color* image (Fig. 7B). Even before processing has been completed on the entire set, this image can be opened in order to check whether the processing is accurate by examining whether the outlines properly identify fibrosis. If not, it is possible to return to the IdentifyObject module and adjust the parameters appropriately, before beginning processing again. Given that there are so many intermediate image processing steps, CellProfiler never saves the processed images unless specifically requested to do so via a SaveImage module. Once processing has been completed, the data for fibrotic area (Fig. 7C) are exported to a tab-delimited text file that can be opened in Excel (Fig. 7D) and then submitted to a statistical analysis program.

Once our pipeline had been validated with test images, the adjusted pipeline with all of the modules described above was used to process a set of six hundred liver tissue images in order to quantify fibrosis after 2, 4, and 6 weeks from bile duct ligation-induced fibrosis in a rat model. Figure 8 illustrates a reduced set of images showing results from the use of CellProfiler to quantitatively evaluate the area occupied by fibrosis at different time points after induction of injury.

5 CONCLUSION

The present paper describes an automatic method for identifying and quantifying a large number of histological images of liver fibrosis. CellProfiler image analysis software provided an objective and reproducible method that precisely identified and quantified the area occupied by fibrous tissue in injured livers at 2, 4 and 6 weeks after bile duct ligation induced-liver fibrosis. CellProfiler simultaneously quantified fibrosis in all images rather than analysing individual images. Using the hardware configuration described in the Material and Methods section, the software ran until all images were analyzed, at a rate of ~10 s/image. The modular and flexible design of the software provided an infrastructure for image analysis that eliminated the many steps which are typically involved in image analysis. Moreover, the resources available at the CellProfiler website, such as tutorials, an on line

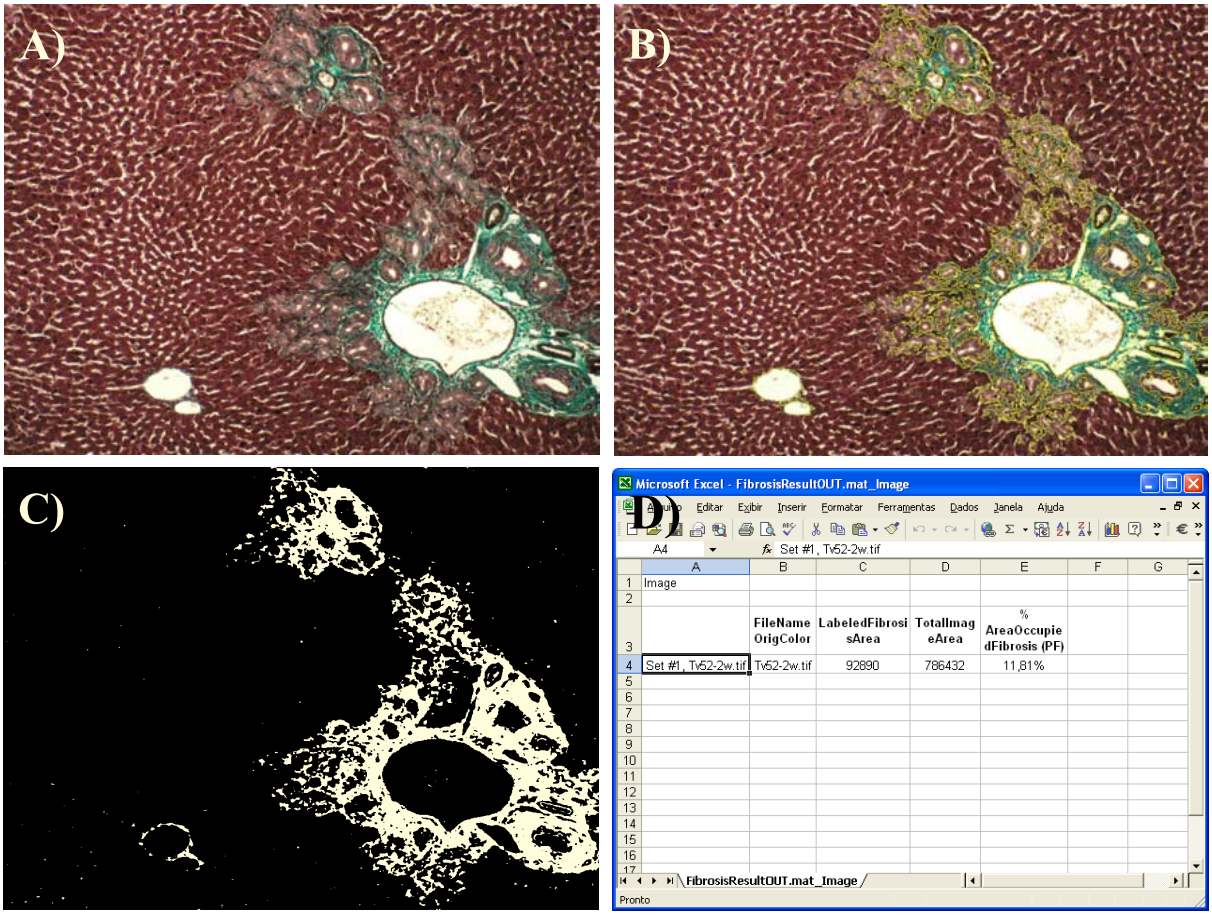

Figure 7 – **A)** Example of the original histological image of liver tissue taken before the image analysis, showing fibrosis stained in green and the parenchymal cells in red; Original magnification 10×. **B)** Image after identification of fibrosis with fibrotic zones outlined in yellow; **C)** Image after measurement of area occupied by fibrosis (stained area): white area is representative of green fibrosis staining; **D)** Result window of the example image analyzed by CellProfiler.

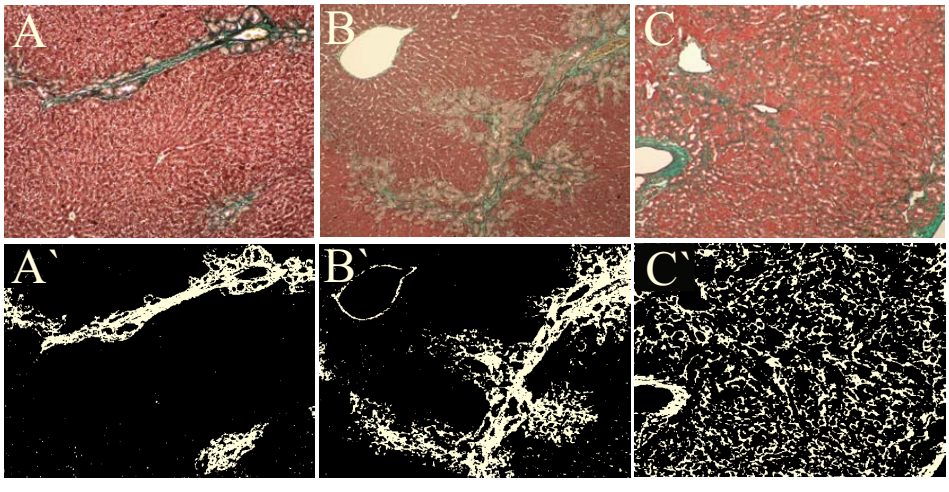

Figure 8 – **(A, B, C)** Representative microscopic image of Masson's trichrome staining at 2, 4 and 6 weeks after bile duct ligation, respectively; original magnification ×10. **(A', B', C')** Corresponding digital images of fibrotic areas quantified by image analysis: white area is representative of green fibrosis staining. Percentage of fibrosis: **A'** = 8.40%, **B'** = 15.35%, **C'** = 21.35%.

discussion forum and example images and pipelines, were all very helpful for the setup of the software for liver fibrosis analysis. This novel tool might be of special value to allow the reaching of valid conclusions regarding the applicability of regenerative therapies for treating liver fibrosis in experimental studies, and also opens the way for further investigations aimed the extending the use of CellProfiler to other tissue assays. A future study involving this approach will entail testing of the effectiveness of placenta-derived stem cell therapy in livers of experimental animal models of fibrosis and cirrhosis.

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