Semiautomated Image Analysis of High Contrast Tissue Areas Using Hue/Saturation/Brightness Based Color Filtering

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Introduction: Quantification and morphological assessment of various tissue elements have numerous applications in fundamental and clinical research. Digital morphometry, in contrast to other morphologic methods, uses personal computers and specific software, to perform precise and highly reproducible results. Additionally, it delivers results in mathematical format. The aim of our study was to develop an open access digital morphometry method for measuring different parameters of various high contrast tissue elements and to elaborate a general work-around for digital morphometry study and data management.

Materials and methods: We used three different types of tissue samples and staining procedures: (1) Diffuse Large B-cell Lymphoma specimens, (2) various stage liver fibrosis specimens and (3) transversely sectioned skeletal muscle tissue to develop a digital morphometric analysis. Image analysis was performed using ImageJ software.

Results: We developed an intuitive and easy to use work-algorithm that fits generic demands. We split the algorithm into three phases, each requiring a different approach and workaround. Using the presented method we were able to quantify the proportion of CD34 positive areas in the DLBCL specimens, the vascularity of this type of lymphoma may be quantified; similarly, this method is optimal for determining the extension of fibrosis in liver specimens; and finally, morphometric analysis of striated muscle fibers was achieved.

Conclusions: We conclude that the use of ImageJ with semiautomatic color segmentation is a reliable and practical way of performing various morphometric measurements. In addition, we are confident that such methods of digital morphometry could have future applications in other areas of pathology and histology.

Keywords: morphometry, image analysis, ImageJ, microscopy, immunohistochemistry

Introduction

Quantification and morphological assessment of various tissue elements have numerous applications in fundamental and clinical research [1]. Digital morphometry, with the use of computer assisted image analysis techniques, in contrast to other morphologic methods, permits the use of personal computers and available software, dedicated or not, to perform precise and highly reproducible results. Additionally, it delivers results in mathematical format. Not surprisingly, many researchers in the field of pathology or other related sciences embraced these techniques and used them in different settings. Some frequently used morphometric parameters include: shape descriptors, descriptors of gray value distribution, descriptors of the size and localization of various image segments and particle count. Introduction of morphometric procedures into research greatly enhanced the reproducibility of various morphometric studies and their results. There are many instances where specific areas of certain tissue sections need to be accurately measured and/or described using various parameters such as dimension, number and ratio to other tissue elements. Digital morphometry spans a wide area of applications and its literature is growing constantly [2–8].

Many methods used by various research groups use proprietary software solutions that sometimes require expensive license. On the other hand, most of these also require advanced computer operating skills and at times even programming skills in various programming environ-

ments, therefore making them even more difficult to access by researchers. We listed some of the proprietary software used in digital image analysis in table I [10–18].

The aim of our study was (1) to develop an open access digital morphometry method for measuring different parameters of various high contrast tissue elements and (2) to elaborate a general work-around for digital morphometry study and data management. The purpose of the method would be to enable the user to quickly and reliably measure the number, size and extension of a given tissue area marked with a specific dye or DAB, using digital microscopic images.

Material and methods

Specimen selection, staining and immunohistochemistry

In order to extend our method's usability we used three different types of tissue samples and staining procedures: (1) Diffuse Large B-cell Lymphoma specimens, (2) various stage liver fibrosis specimens and (3) transversely sectioned skeletal muscle tissue (see Figures 1–3). For each type of lesion/tissue a number of 20 cases were selected.

All histological sections were fixed in 4% neutral formaldehyde solution and later paraffin-embedded. The approximately 3–5 μm thick sections were deparaffinised by xylene-ethanol sequence. Diffuse Large B-cell Lymphoma specimens were selected from a previous study and vascular elements were highlighted using CD34 an-

Table I.   Some of the software used in digital image analysis by various researchers

Author	Application	Software	Vendor	Ref
Garton et al. (2010)	Skeletal muscle fiber analysis	MetaMorph	SPOT Imaging Solutions (Diagnostic Instruments, Inc.) Sterling Heights, MI, USA	10
Garton et al. (2010)	Skeletal muscle fiber analysis	ImagePro Plus 2.0	Media Cybernetics, Silver Spring, MD	10
Krajewska et al. (2009)	Cell death and fibrosis assessment	ImageScope	Aperio Technologies, Inc., USA	11
Hall et al. (2008)	HER2 analysis	Matlab	The MathWorks, Inc. Natwick, MA, USA	12
Ficsor et al. (2008)	Colon	Microsoft Visual C++	Microsoft	13
Caruntu, Covici (2006)	Renal corpuscle	Zeiss KS400	Carl Zeiss Jena GmbH, Jena Germany	14
Weaver et al. (2003)	Sentinel Lymph Node Micrometastases in Breast Cancer	ChromaVision Automated Cell Imaging System	ChromaVision Medical Systems, Inc., San Juan Capistrano, CA , USA	15
Căruntu et. al (2002)	Dental tissue	Zeiss KS400	Carl Zeiss Jena GmbH, Jena Germany	16
Lehr et al. (2001)	HER 2 analysis	Photoshop	Adobe Systems Incorporated, San Jose, CA, USA	17
Francis et al. (2000)	Breast carcinoma	CAS-200	Becton Dickinson, Elmhurst, Illinois, USA	18

tibody (Class II, clone QBEnd 10, Dako, Denmark). Application of the primary antibody was followed by the two-step peroxidase technique. The staining process was achieved using DAB chromogen. Various stage liver fibrosis specimens were stained using Masson's trichrome stain and were obtained from another study regarding liver fibrosis. Finally, transversely sectioned skeletal muscle tissue samples were obtained from gastrocnemian muscles of AKR mice.

Image acquisition
Digital slides were made with Zeiss MiraxScan digital slide acquisition system (Carl Zeiss Jena GmbH, Jena Germany) mounted with Marlin F-146C digital camera at a resolution of 1392×1040 pixels with Sony ICX267 sensor (Allied Vision Technologies GmbH, Stadtroda Germany) through Zeiss Plan-Apochromat 20× magnification and 0.8 numeric aperture. The acquisition system was controlled by MiraxScan software (3DHistech, Budapest, Hungary) installed on a Fujitsu-Siemens Celsius Workstation computer (4 × 2 GHz CPU, 2 GB RAM).

For each tissue type, a number of 5 images / specimen were saved from the digital slides both in TIFF (Tagged Image File Format) and JPG (Joint Photographic Experts Group) format using the 3DHistech Panoramic Viewer.

Thus, a total number of 300 digital images were prepared for the study. Digital images were not altered in any way (contrast, luminosity, levels etc.) before morphometric analysis, i.e. they were further used as they were saved by the Panoramic Viewer software.

It should be noted that digital image acquisition in our laboratory indeed uses proprietary tools as opposed to our main goal to develop an open source solution. Nevertheless, digital microscopic images may be obtained in many ways and it varies greatly among different pathology laboratories where such studies are being performed.

Image processing
Image analysis methods were performed using ImageJ software, version 1.44c (developed by Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) [19] and Java 1.6.0_20 (32-bit) engine. We deliberately choose this software since it is freely available and is platform independent. Additionally, we used the color thresholding image adjustment employing the Threshold_Colour plugin (now included in the ij.jar). This plugin is a modification of Bob Dougherty's BandPass2 filter [20]. All image processing was performed on a DualCore (2 × 2.2 Ghz), 2 Gb memory PC running alternatively Microsoft 7 and Ubuntu 10.10 (Linux Kernel 2.6.35-28).
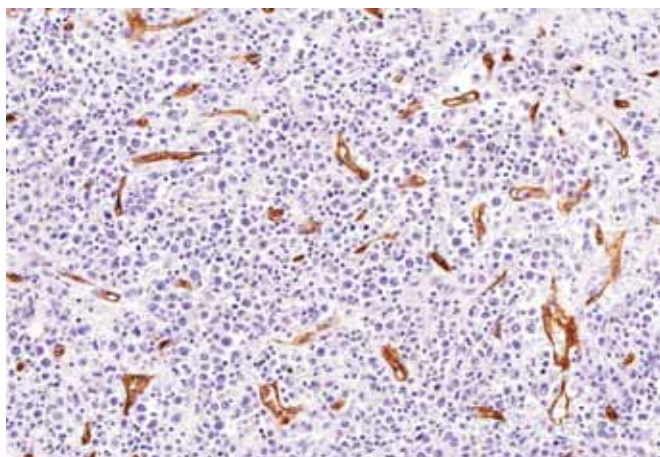
Fig. 1.   Tissue specimen from a DLBCL case marked for CD34 antibody, immunohistochemistry, DAB chromogen
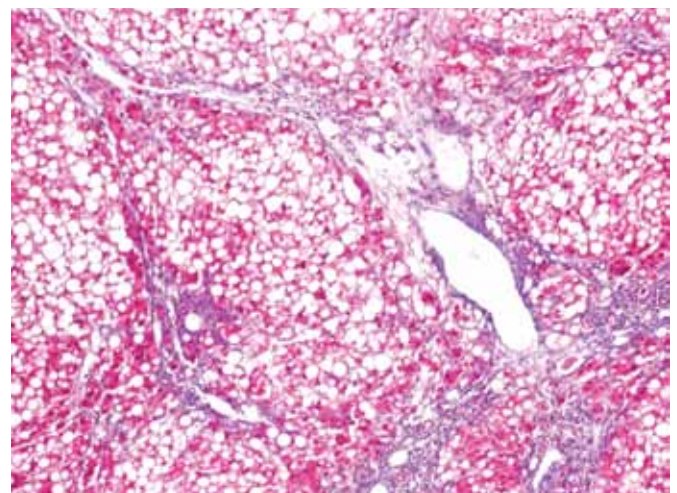
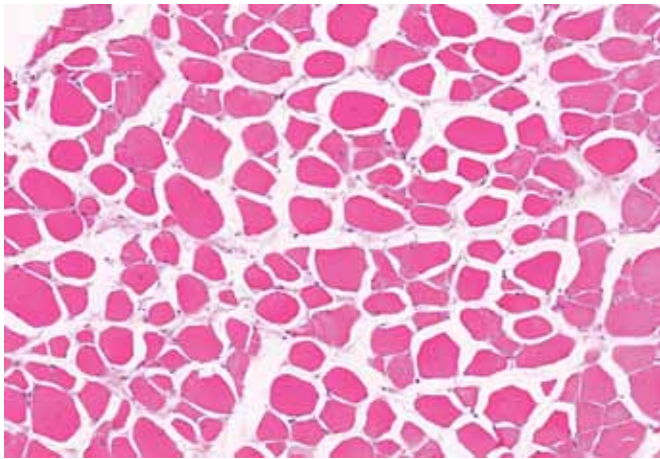**Fig. 2.   Liver tissue section stained with trichrome stain**

Fig. 3.   Mouse skeletal muscle fibers (transverse section), haematoxylin-eosin stain

Fig. 4.   Visual representation of the phases of digital image analysis of our study

Results. Description of the method

In order to achieve the desired goals we found the following work-algorithm most lucrative and best fit for generic digital morphometric studies (see Figure 4) as well as for our aims. We split the algorithm into three phases, each requiring different approach and workaround.

Phase one is the most frequently overlooked, however, we consider it the most critical amongst the other work phases in any digital morphometric study. It represents the moment when one should turn off the computer a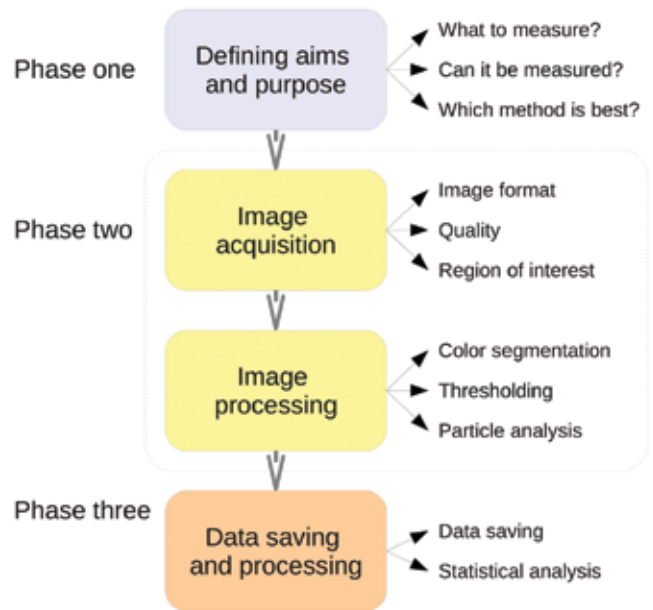nd instead look into the microscope and carefully determine what can possibly be measured using digital morphometry. Our method requires that the measured areas be of high contrast and have significantly different hues. Keeping this in mind helps the user to minimize error and quantification of parameters that in fact cannot be measured. This is the reason why we specially developed this method for im-
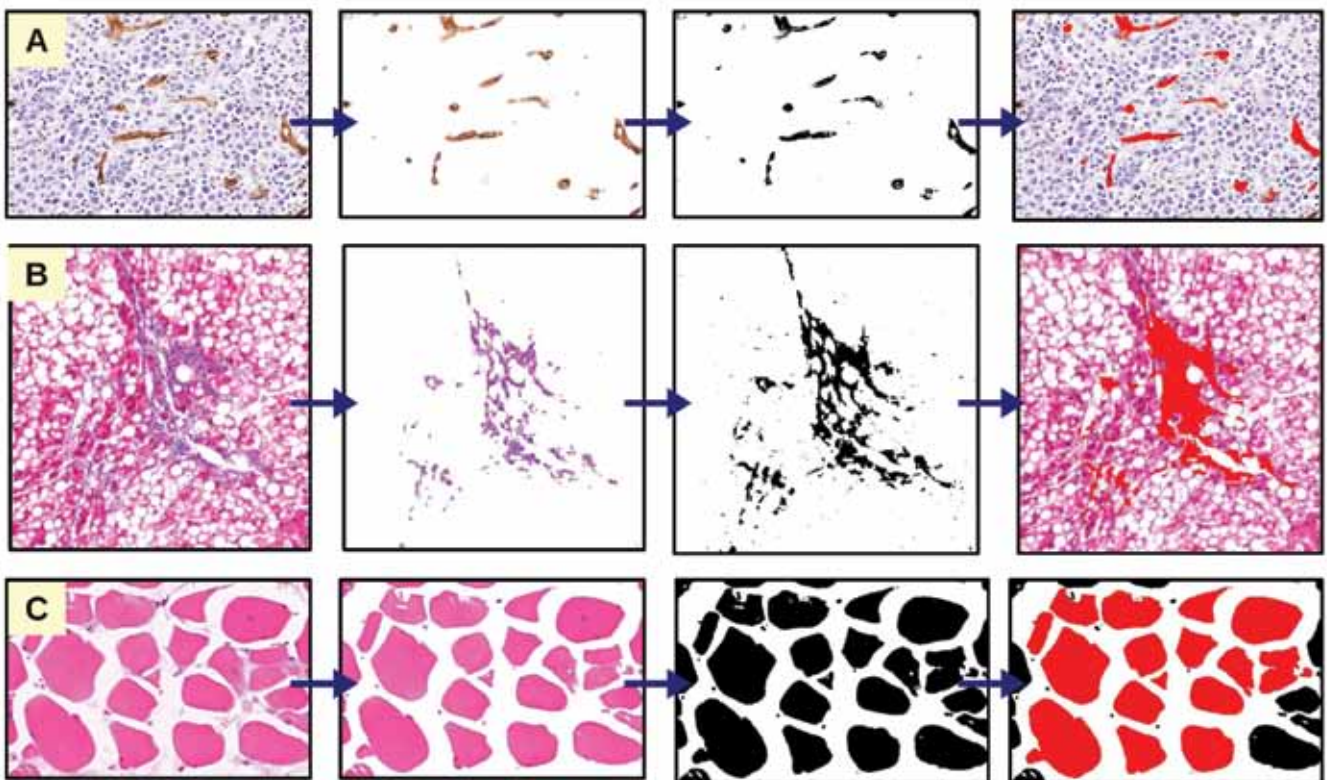

Fig. 5.   The effects of digital image processing on various tissue samples using color segmentation, thresholding (A: Diffuse Large B-cell Lymphoma specimens with IHC staining for CD34 antibody; B: liver fibrosis specimen; C: transversely sectioned skeletal muscle tissue sample). Note: the last column of images are mounted images to show what was actually measured.
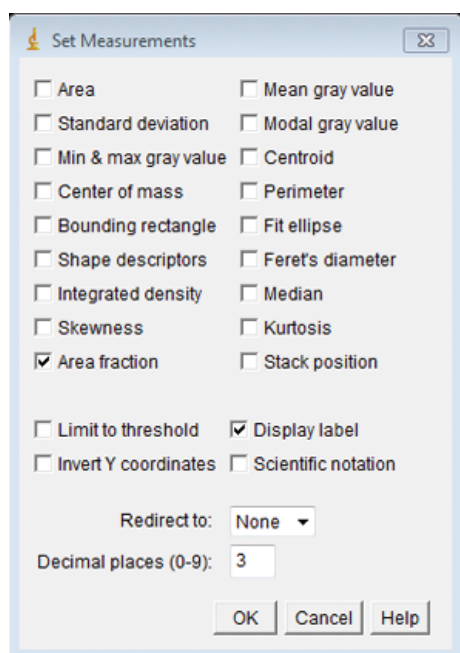
Fig. 6.   ImageJ's Set Measurements settings panel

Fig. 7.   ImageJ's Analyze Particles initial setting panel

munohistochemical DAB (3,3'-Diaminobenzidine) stain, Masson's trichrome stain and selected cases of haemato-xylin-eosin stain. It is hard to establish a list of applications that can be used with our method since it can be highly customized. Nevertheless it is important to set from the beginning those parameters that are to be measured; these parameters may include: specific object area, color and number.

Phase two includes the digital image acquisition and morphometric procedures. Image acquisition is important since we found that it can significantly influence the outcome of the results. During image acquisition the examiner arbitrarily picks usually up to 5 digital images per tissue section to have digital morphometry performed on. It is obvious that these images have to be chosen very carefully in order to best describe the tissue section as a whole. In this regard a thorough analysis of the whole tissue section is necessary by visual inspection in order to be able to choose those fields that best describe the entire tissue. In the case of DLBCL with CD34 IHC labeling it is relatively easy to pick such fields since the whole lymph node is equally affected by the disease and the analysis of different regions will have similar results. In case of liver fibrosis inspection of the whole tissue sample is mandatory in order not to over- or underestimate the extension of fibrosis. Regarding striated muscle fibers the cave out is to choose fibers that are perfectly perpendicular to the sectioning plane.

Morphometric procedures comprise several steps that include HSB-based color segmentation and particle analysis. The end result is the analysis of those areas that were "separated" (through color segmentation) from the original tissue based on their HSB characteristics. The typical image processing steps are presented in Figure 5. In the following section we describe the step-by-step procedure.
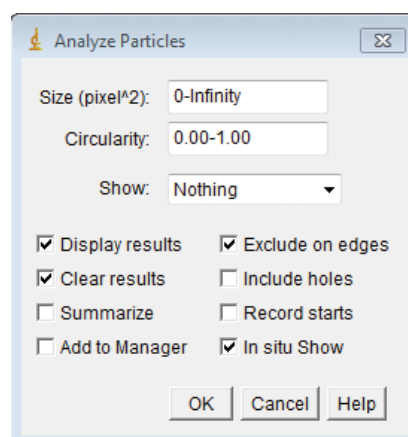
After launching ImageJ picture editor and processing tool, the desired microscopic digital image is opened either by `File > Open` or by simply dragging the image to ImageJ's toolbar. Once the image is open it may be segmented based on HSB parameters using `Image > Adjust > Color Threshold`. (Note that this feature is relatively new to ImageJ, earlier versions needed to install the additional Threshold_Colour plugin). A separate window appears displaying three histograms default for Hue, Saturation and Brightness. Using this window the user may filter those areas that need to be measured. Filtering is achieved by setting the minimum and maximum for the HSB filters and specifying whether a specific filter passes or stops (Pass/Stop) a certain spectrum range. For the three chosen specimens we found the settings presented in Table II to be typical for color thresholding. After the desired areas remain visible and the rest of the tissue obscured by pressing `Threshold` on the Threshold Color window all filtered areas are displayed in foreground color (typically black) and non-filtered areas as background color (typically white). Foreground/background color settings may be changed accessing `Edit > Options > Colors`. Thresholding is a key step in HSB-based image processing since it is up to the user to set which tissue areas remain visible and remain obscure. We found that best results are obtained using thin sections and high quality images. After thresholding is performed, in order for the next step to be applicable, the resulted image must be converted to 8-bit format (`Image > Type > 8-bit`), regardless of initial format. Before particle count the user must set which parameters are to be measured (`Analyze > Set Measurements`), see Figure 6. For typical morphometric analysis

Table II.   Typical settings for color thresholding for the three tissue samples, numbers indicate minimum and maximum values while letters in brackets indicate filter type (P – pass; S – stop)

Tissue and stain	Hue	Saturation	Brightness
DLBCL (DAB)	44/255 (S)	37/255 (P)	0/255 (P)
Liver (Masson's trichrome)	0/216 (P)	56/255 (P)	0/255 (P)
Muscle (haematoxylin-eosin	0/217 (S)	112/255 (P)	0/255 (P)

area, area fraction, perimeter, Ferret's diameter will suffice. After marking the desired measurements, particle analysis may be performed on the thresholded image (`Analyze > Analyze Particles`), Figure 7. The initial panel has some key settings worth mentioning. Size limits the size of the particles measured. If the thresholded image contains too much background noise or „grain", setting minimum particle size other than zero will exclude much of this noise. The minimum particle size to be excluded is best determined empirically. For optimal results Display results, Clear results, Summarize, Exclude on edges must be checked. This part of the image processing is entirely automatic since particle count and analysis is performed by ImageJ.

Phase three consists of data saving and management and begins after particle analysis is performed. After performing phase two of the image analysis, all filtered tissue areas measured (and their parameters) and included in a tabulated data format. ImageJ displays the results in a separate window (`Results`) that may be saved in one of the popular data storage file formats (e.g. csv or xls). This data is then available for further statistical analysis and data mining.

Using the presented method we were able to quantify the proportion of CD34 positive areas in the DLBCL specimens. Assuming that most CD34 areas correspond to endothelial structures, the vascularity of this type of lymphoma may be quantified. Similarly, this method is optimal for determining the extension of fibrosis in the examined liver specimens. Finally, the perimeter and Ferret's diameter were measured for the striated muscle fibers. These parameters are used to compare changes in muscle fiber diameter after drug administration to ARK mice.

Discussions

In our paper we presented a practical approach and work-algorithm to perform Hue-Saturation-Brightness based image analysis of high contrast tissue components. We have chosen a software (ImageJ) that is freely available along with extensive documentation [19,20]. This software is platform independent, therefore it may be used on any operating system that is bundled with Java Engine. Additionally we presented the required steps in order to perform the method. As we stated in the introduction, our goal was to develop an accessible work-flow devoid of too many technical terms so that advanced programming knowledge is not a requirement. This method is by far not intended to provide an all-in-one image analysis tool, but rather a starting point in performing more complex digital image morphometric analysis. Its ImageJ based core methodology renders this method highly customizable and adaptable to various demands. In the spirit of open source we provide additional information and also the complete set of functions and procedures of phase two on our continuously updated webpage, located at pathologia.ro/morphometry.

Conclusions

We conclude that the use of ImageJ with semiautomatic color segmentation is a reliable and practical way of performing various morphometric measurements. In addition, we are confident that such methods of digital morphometry could have future applications in other areas of pathology and histology. This is mainly due to the fact that the method that we presented uses an open source software environment that is available to everyone and extensive documentation exists on the Internet.

Acknowledgement

Abbreviations

DLBCL – Diffuse Large B-cell Lymphoma
DAB – 3,3'-Diaminobenzidine
IHC – Immunohistochemistry
HSB – Hue-Saturation-Brightness

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