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Review of free software tools for image analysis of fluorescence cell micrographs

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Summary

An increasing number of free software tools have been made available for the evaluation of fluorescence cell micrographs. The main users are biologists and related life scientists with no or little knowledge of image processing. In this review, we give an overview of available tools and guidelines about which tools the users should use to segment fluorescence micrographs. We selected 15 free tools and divided them into stand-alone, Matlab-based, ImageJ-based, free demo versions of commercial tools and data sharing tools. The review consists of two parts: First, we developed a criteria catalogue and rated the tools regarding structural requirements, functionality (flexibility, segmentation and image processing filters) and usability (documentation, data management, usability and visualization). Second, we performed an image processing case study with four representative fluorescence micrograph segmentation tasks with figure-ground and cell separation. The tools display a wide range of functionality and usability. In the image processing case study, we were able to perform figure-ground separation in all micrographs using mainly thresholding. Cell separation was not possible with most of the tools, because cell separation methods are provided only by a subset of the tools and are difficult to parametrize and to use. Most important is that the usability matches the functionality of a tool. To be usable, specialized tools with less functionality need to fulfill less usability criteria, whereas multipurpose tools need a well-structured menu and intuitive graphical user interface.

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Introduction

Experiments with fluorescence micrographs in biology, microbiology, immunology, virology or related disciplines most often require the evaluation of a large number of micrographs depicting cells and cellular structures. A manual examination, assessment and analysis of data with increasing complexity and volume is prone to inter- and intraobserver errors and is a time-consuming task. Due to limitations of resources, usually, only a minimal subset of the depicted cells is analyzed manually, even though much more image data are available for most experiments. Automatic and semiautomatic image analysis methods enhance reproducibility and objectivity of such image-based experiments and support the analysis of more than a couple of cells manually.

Different approaches addressing the problem of (semi-) automatic cell segmentation and analysis of fluorescence micrographs have been proposed. Moreover, some of these approaches have been made available to the community in order to provide tools for various fluorescence image analysis problems as well as to support the ongoing research of the community. Wang *et al.* (2009) listed free tools, such as CellProfiler (Carpenter *et al.*, 2007), Cell-ID (Chernomoretz *et al.*, 2008) , CellTracker (Du *et al.*, 2010), ImageJ (Girish & Vijayalakshmi, 2004; Collins, 2007) and GoFigure (Gelas *et al.*), all designed for the segmentation of cells and subcellular structures in fluorescence micrographs. They did not rate the tools. Also, there are websites (Ospedale San Raffaele) that list existing software tools for fluorescence micrograph image analysis, covering image segmentation, cell analysis, registration or deconvolution. Nevertheless, the websites describe the listed tools quite briefly, if at all, and do not compare them. To our knowledge, there are no comparisons of free image analysis tools with regard to their applicability to cell fluorescence micrographs and with respect to various segmentation and analysis tasks.

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Hence, the selection of an adequate software tool for fluorescence micrograph analysis remains a challenging problem.

Therefore, this paper provides a systematic review and comparison of free software tools for the segmentation and analysis of cell fluorescence micrographs. The review consists of two parts: an evaluation of the tools based on a self-developed criteria catalogue and an image processing case study. The evaluation was motivated by and done similarly to the study by Liao *et al.* (2008), who selected, evaluated and compared free DICOM reading and visualization software packages. We further classified each criterion, to either contribute to the usability or functionality of a tool and evaluated usability against functionality. As it is hard to quantify functionality and even hard to quantify usability, this evaluation can only give a coarse idea of the quantitative evaluation of usability against functionality.

For a qualitative evaluation of usability and functionality, we performed a user case study that has to evaluate the required effort and performance of the segmentation of four representative fluorescence micrographs. The segmentation tasks consist of figure-ground and cell separation. Our experiments allow us to provide future users with guidelines for the selection of software tools that ease fluorescence micrograph evaluation, rather than complicating it.

Materials

In the following section, we state how we selected the tools in this evaluation and how we divided them to be examined into five functional groups.

Selection criteria

We included image analysis tools in the evaluation if they fulfilled all of four selection criteria: (1) The tool is explicitly designed for the evaluation of microscopic images and can handle fluorescence micrographs. (2) The tool is free to download and use on a PC. (3) The tool provides a graphical user interface (GUI). (4) The tool can be used without programming experience. We additionally included ImageJ (Collins, 2007) and Fiji (Schindelin *et al.*, 2012) in the evaluation, which do not fulfill criteria (1) but are used for fluorescence image analysis. An overview of the selected and examined tools is given in Table 1. Altogether, there are 15 tools available that fulfill the selection criteria.

Functional groups

The examined tools can be divided into five groups, depending on their design and implementation.

Tool Group I – stand-alone tools. This group consists of stand-alone tools dedicated to the assessment and segmentation of varying fluorescence micrographs with an intuitive GUI and filters for preprocessing and manual, interactive and automated segmentation. The respective tools are Icy, CellProfiler, Daime and Color Segmentation (ColorSeg.).

Icy was developed and is maintained at the Quantitative Image Analysis Unit, Institute Pasteur by de Chaumont *et al.* (2011). It comes with native plug-in support, a comprehensive website, and facilitates the development and usage of a variety of image processing algorithms. Figure 1(A) shows a screenshot of the GUI.

CellProfiler was developed by Anne Carpenter's team at the Broad Institute, MIT and Harvard (Carpenter *et al.*, 2007). It was initially designed for the evaluation of high-throughput experiments. Therefore, the control concept differs from other tools. The user designs his own image processing pipelines from modules that encapsulate functionality from image processing to object detection and object analysis (Fig. 1B). CellProfiler supports ImageJ plug-ins (Kamentsky *et al.*, 2011). The GUI – designed by a biologist – considers the specific requirements of inexperienced users.

Daime was developed by Daims *et al.* (2006) at the Department of Microbial Ecology, University of Vienna. It is specialized for the analysis of fluorescence *in situ* hybridization (FISH) probes. It provides thresholding and edge detection for the segmentation of two- or three-dimensional cellular objects in single images, image series or stacks. The user can analyze features of the segmented objects and quantify spatial localizations of microorganisms (Daims & Wagner, 2011). See Fig. 1(C) for a screenshot of the GUI.

ColorSeg. was developed at the Center for Microbial Ecology Image Analysis System (CMEIAS), Michigan State University by Gross *et al.* (2010). CIMEAS provides two stand-alone applications for image processing. The first tool, ColorSeg., is an interactive segmentation tool that uses colour information and spatial relationships between user-selected pixels for the segmentation of the remaining pixels (Fig. 1D). The second CMEIAS tool provides basic image processing methods and classifies cells of the most predominant bacterial morphotypes (Liu *et al.*, 2001).

Tool Group II – Matlab-based tools. The second group is written in the Matlab scripting language and contains BlobFinder and CellSegmenter. Both provide a clear GUI with specialized segmentation functionality.

BlobFinder was developed at the Centre for Image Analysis, Uppsala University by Allalou & Wählby (2009). It uses the following workflow for the segmentation of subcellular structures in fluorescence micrographs: first, cell nuclei segmentation with Otsu thresholding; second, cytoplasm estimation with distance transform and a user-defined cell radius; and third, detection of bright spots assigned to a cell. There are two modes available in the BlobFinder tool, the detection of spots per nucleus on the whole image or spots per nuclei on a cellular level.

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Table 1. Evaluated tools with version number, download location, and if a registration is necessary

Tool Group & Name		Version	URL Registration necessary	
I	Icy	1.1.0.0	www.bioimageanalysis.org/icy	
	CellProfiler	2.0	www.cellprofiler.org	
	Daime	1.3.1	www.microbial-ecology.net/daime	√
	ColorSeg.	1.0	www.cme.msu.edu/cmeias	
II	BlobFinder	3.2	www.cb.uu.se/~amin/BlobFinder/	√
	CellSegmenter	none	arep.med.harvard.edu/QMS	
III	Fiji	20110307	Fiji.sc	
	MiToBo	0.9.6	www.informatik.uni-halle.de/mitobo	
	ImageJ	1.45	rsbweb.nih.gov/ij	
IV	Volocity Demo	5.5.1	www.perkinelmer.com	√
	AxioVision LE	4.8.2	www.zeiss.de/axiovision	√
	LAS AF Lite	2.6.0	ftp://ftp.llt.de/softlib/LAS_AF_Lite	
V	Omero	4.3.1	www.openmicroscopy.org	
	Bisque	0.5.0	bisque.ece.ucsb.edu	

CellSegmenter was developed at the Department of Genetics, Harvard Medical School by Bakal *et al.* (2007). It uses a single-channel grey-scale image and obtains an initial segmentation by adjusting a user-defined threshold to the current image. The segmentation can be refined with user-defined barriers. In contrast to the stand-alone tool BlobFinder, CellSegmenter needs a valid Matlab license.

Tool Group III – ImageJ-based tools. The third group pools ImageJ and the ImageJ-based tools MiToBo and Fiji. All three offer a great deal of functionality and are extendible.

ImageJ was developed with a grant from the National Institutes of Health by Schneider *et al.* (2012). It is an open-source platform for image processing and is used in a variety of research areas in medicine and biology. The user community is large and growing, with many people developing their own plug-ins. Other tools, such as CellProfiler and Icy, have started to support and utilize ImageJ plug-ins. Figure 1(E) shows the GUI of ImageJ.

MiToBo that is an abbreviation for microscope image analysis toolbox was developed at the Martin Luther University of Halle-Wittenberg by Möller & Posch (2013). It is based on the autodocumentation system Alida (Möller *et al.*, 2011) and uses various plug-ins, for example, for microscopy cell image analysis (Möller & Posch, 2011) and the evaluation of cell motility (Glaß *et al.*, 2011).

Fiji, an open-source project, was developed to facilitate the usage of ImageJ with, for example, automatic updates and comprehensive documentation by Schindelin *et al.* (2012) and incorporates a large set of various image analysis and cell segmentation methods. Image processing and segmentation functionality are directly integrated into the ImageJ menu structure.

Tool Group IV – free demo versions of commercial tools. The fourth group covers the free demonstration and evaluation versions of commercial image processing tools with restricted functionality. This group includes Volocity Demo, AxioVision LE, and LAS AF Lite.

Volocity Demo is provided by Perkin Elmer as a demo version for the 3D imaging software Volocity (PerkinElmer). It comes with a full image processing scope, but restricts the export of results. See Figure 1(F) for a screenshot of the GUI.

Zeiss distributes AxioVision LE as a demo version of the Axio Vision software (Carl Zeiss Microscopy GmbH). The demo version includes basic image processing functionality and manual segmentation functionality (Fig. 1G).

LAS AF Lite is a free version of the Leica Application Suite Advanced Fluorescent (LAS AF, Leica Microsystems GmbH) and is highly restricted in functionality to a mere viewer (Fig. 1 H). As the demo version of LAS AF does not include any means for segmentation, we include LAS AF Lite in Table 2, but exclude it from further evaluation.

Tool Group V – data sharing tools. The fifth group bundles image analysis tools with a web-based client-server architecture and focuses on the organization, assessment and distribution of image data. They provide web tools for manual segmentation and analysis of fluorescence micrographs. The group consists of Omero and Bisque.

Omero was developed at the University of Dundee, the National Institute of Aging, the Harvard Medical School and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison by Swedlow *et al.* (2009). It provides image processing, segmentation with scripts (Johnston *et al.*, 2006) and a web interface for manual segmentation.

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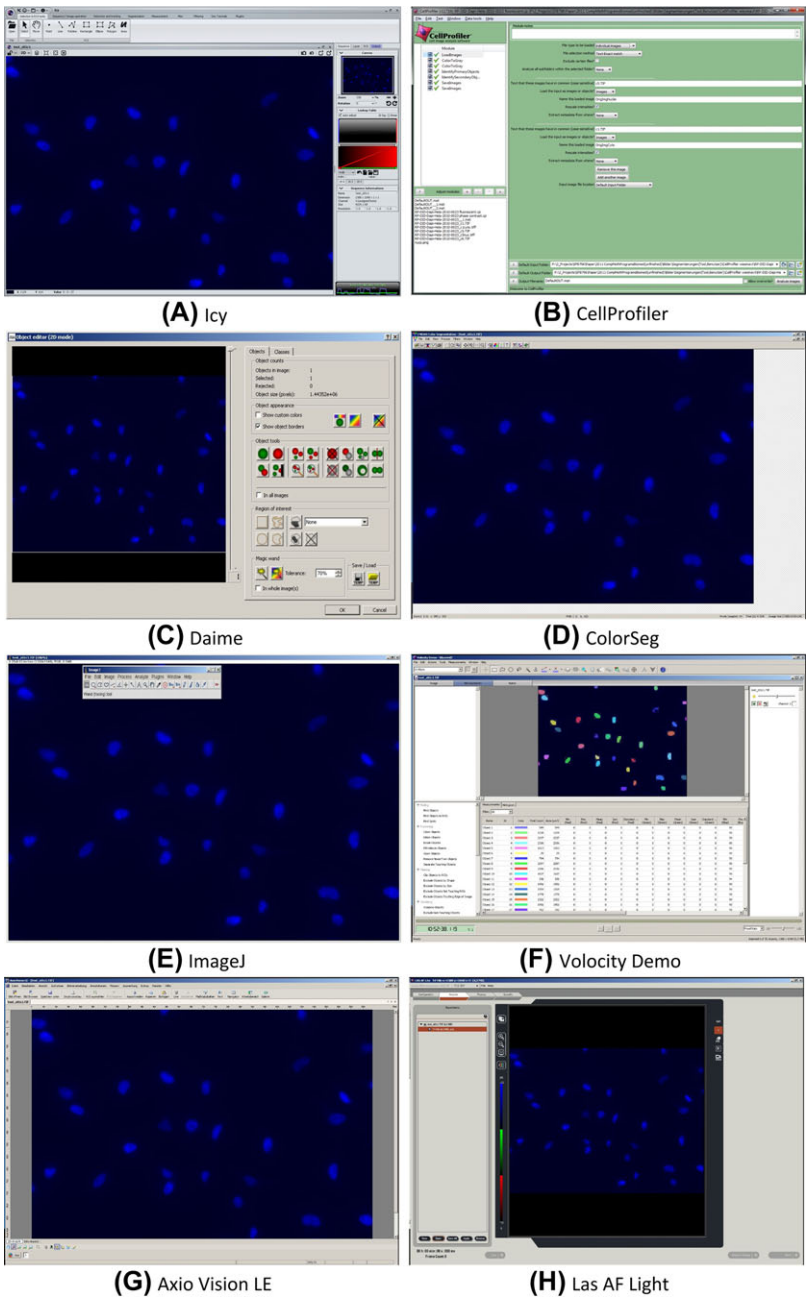

Fig. 1. GUIs of different tools.

Bisque was developed at the Center for Bio-Image Informatics, University of California by Kvilekval *et al.* (2010). It is specifically designed for 5D image data and integrates manual segmentations and fixed workflows for specific image analysis tasks (e.g. 3D nuclei detector).

Methods

This section will present our criteria catalogue and the design of an image processing case study.

Criteria catalogue

We have developed a criteria catalogue to assess the structural requirements, usability and functionality criteria, which range from software documentation to program usage, with a focus on image processing and cell segmentation.

Structural requirements include computer hardware needed, working memory usage, disk space requirements and the installation procedure. Usability criteria include documentation, data management, usability, flexibility, visualization,

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Table 2. Set of review criteria and availability for the evaluated tools

		I				II		III		IV			V	
Criteria Group . . .	Tool Group	Icy	CellProfiler . . .	Daime . . .	ColorSeg . . .	BlobFinder	CellSegmenter	ImageJ/Fiji	MiToBo	Volocity Demo	AxioVision LE	LAS AF Lite	Omero	Bisque
A	Handbook	√	√	√	√		√	√	√	√	√	√	√	
	Support	√	√		√			√	√				√	√
	Source Code	√	√	√			√	√	√				√	√
B	Bio-Formats Import							√	√				√	√
	Export	√	√		√			√	√	√				
C		√	√		√	√	√			√	√		√	√
	Intuitiveness													
	Wizards		√							√				
D	Histogram		√	√				√	√		√		√	
	Adj. Display	√						√	√	√	√	√	√	
E	Macros		√					√	√	√			√	
	Plugins	√	√					√		√				
F	Manual	√	√					√	√	√	√		√	√
		√	√		√		√	√	√	√				
	Interactive													
		√	√	√	√	√	√	√						
	Automated													
G		√	√	√	√			√		√	√			
	Preprocessing													
		√	√	√	√	√	√	√	√	√				
	Thresholding													
	Advanced	√	√					√	√	√				

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segmentation and image processing filters. The corresponding terms to Table 2 are named in parentheses.

Documentation (Criteria Group A). Software documentation helps users to orient themselves to a software tool or answer-specific questions. It can be organized with a written manual (Handbook) or electronic support via e-mail, a forum or a Wiki (Support). Some tools also provide the source code, which facilitates the software's use by computer-science-experienced users (Source Code).

Data management (Criteria Group B). Data management includes loading and saving fluorescence micrographs and segmentation results. File formats for fluorescence micrographs range from proprietary formats from commercial distributors to open-source solutions. LOCI Bio-Formats (LOCI) support all common image formats such as TIFF, PNG, JPEG, BMP, GIF and RAW (Miano, 1999) and many vendor-specific microscopic image formats, including the Zeiss CZI-, the Leica LSM- and the Perkin Elmer MVD2-Format (Bio-Formats Import). For postprocessing, publication or archiving of the (intermediate) results, the user needs to export them as an image-, XML- or text-file (Export).

Usability (Criteria Group C). Almost all users who evaluate fluorescence micrographs are experts in biology or a related fields in life science but are most likely inexperienced with image processing or even programming details. We evaluate whether this fact is considered in the design of the GUI and the menu structure of the tools. Based on our criteria, a tool is intuitive if the GUI is self-explanatory and the menu structure meets the expectation of a life scientist (Intuitiveness). Additionally, some tools provide guidance through the image processing tasks at hand (Wizards).

Visualization (Criteria Group D). The computation and display of a histogram of image intensities (Histogram) and the option to modify brightness and contrast (Adj. Display) is important for the display of fluorescence micrographs, because their signal intensities can vary. As we focus on 2D segmentation, we will not include 3D visualization functionality.

Flexibility (Criteria Group E). Some tools offer increased flexibility to users who are more experienced in image processing or computer science. Macros, a standardized sequence of image processing steps, and batch processing enable users to analyze large amounts of image data (Macros). With built-in plug-in support, users can add specialized functionality and adapt the tool to their specific problems or, with custom filter support, design and integrate their own filters (Plug-ins).

Segmentation (Criteria Group F). Segmentation methods can be divided into manual, interactive or automated. Manual segmentation requires the user to delineate every single cell on each micrograph (Manual). Interactive methods require less user interaction (Dach *et al.*, 2011) and compute parameters for algorithms on the basis of user input (Interactive). Automated algorithms can be applied to various segmentation algorithms without any user interactions (Automated).

Image processing filters (Criteria Group G). Most segmentation methods are composed of preprocessing and segmentation filters. Micrographs can be enhanced by adjusting brightness, contrast, gamma value or – as fluorescence micrographs may have a low signal to noise ratio – image enhancement filters such as denoising or edge enhancement (Preprocessing). The most important segmentation methods for fluorescence micrographs are thresholding (Thresholding) and advanced methods such as watershed transformation or level sets (Advanced).

Image processing case study

To evaluate functionality and usability of the selected tools qualitatively, we performed an image processing case study with a test user. The test user has a life science background, is experienced with fluorescence micrographs, has an appropriate knowledge with computers and software tools and has little knowledge in image processing. The test user was asked to segment four fluorescence micrographs (FM1-4) with all of the tools. Figure 2 displays the four fluorescence micrographs (FM1-4). They have been acquired with the following parameters:

FM1 shows nuclei of HeLa cells, stained with DAPI and acquired with a Zeiss Axiovert Microscope and 20× oil objective. The resolution is 1388×1040, the scale 40μm.

FM2 shows cytoplasmic membranes of HeLa cells, stained with DiD and acquired with a Zeiss Axiovert Microscope and 20× oil objective. The resolution is 1388×1040, the scale 40μm.

FM3 shows HeLa cells infected with Salmonella Typhimurium, stained with anti-Lamp1 and aquired with a Zeiss LSM 700 and 63× oil objective, The resolution is 1388×1040, the scale 15 μm.

FM4 shows L929 cells, stained with FDA and aquired with a Olympus IX71 and 20× air objective. The resolution is 1376×1032, the scale 40 μm.

Generally, a cell segmentation task can be divided into two subtasks: figure-ground and cell separation. The four studied micrographs displayed varying levels of difficulty regarding figure-ground and cell separation: (FM1) figure-ground separation easy, cell separation none, (FM2) figure-ground separation hard, cell separation easy, (FM3) figure-ground separation hard, cell separation hard, (FM4) figure-ground separation easy, and cell separation hard.

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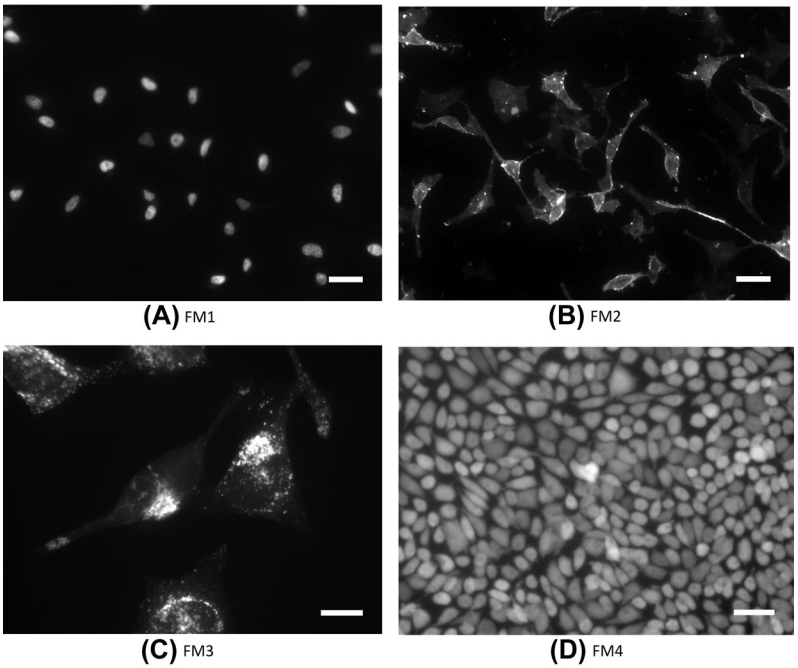

Fig. 2. Contrast-enhanced fluorescence micrographs for the evaluation of the image processing tools.

Although figure-ground separation for FM1, FM2 and FM4 could be performed with global thresholding, in FM3, the intracellular contrast was higher than the contrast to the background and the segmentation needed to integrate local image information. For FM1, no cell separation was necessary. Cell separation for FM2, FM3 and FM4 could be achieved with locally adaptive thresholding or advanced methods, such as watershed or level sets.

The test user performed the segmentation tasks in a predefined order starting with fluorescence micrograph FM1; for cell separation in FM2, the segmented nuclei from FM1 could have been used (Figs. 2A, B). Afterwards fluorescence micrographs FM3 and FM4 were segmented (Figs. 2C, D). We deliberately gave no guidance during the segmentations: The test user could perform the segmentation tasks either manually, interactively or automatically. This leads to segmentation results that reflect the segmentation performance of a typical user with the tested tools. This result often might not be the best achievable result with a tool.

Results

This section presents the tools' evaluation with respect to our criteria catalogue and the results of the image processing case study.

Criteria catalogue

We evaluated the tools with respect to, first, structural requirements and, second, functionality and usability criteria.

First, structural requirements consider the installation of the tools. All tools can be installed on Microsoft Windows XP and Windows 7 ('Selection criteria' section), and all stand-alone, ImageJ-based and extended data-sharing tools (Tool Groups I, III and V from 'Functional groups' section) provide a Linux as well as a MacOS version. Daime provides an additional Linux version. The requirement for disk space ranges from 3B (CellSegmenter) to 432 (LAS AF Lite) and is not critical for all software tools.

Working memory consumption after software tool start ranges from 2 MB for ColorSeg to 350 MB for CellSegmenter which requires MatLab. The totally used memory depends on the underlying datasets. The evaluation of standard datasets with around 30 images tuples consisting of two to five channels is possible with each tool.

Icy and ImageJ additionally need the Java Runtime Environment; BlobFinder is provided together with the Matlab Runtime Environment, which needs an additional 260 MB of hard disk space; MiToBo and Omero need additional libraries, which require additional disk space and CellSegmenter needs a valid Matlab license. All native Windows' tools such as CellProfiler, Daime, ColorSeg. and CellSegmenter and all tools from Tool Group IV (demo version of commercial tools) are provided with an intuitive installer, and thus, can be easily installed. Icy, ImageJ, MiToBo and Fiji are based on the Java programming language and do not need to be installed but need an existing installation of the Java Virtual Machine. MiToBo is not provided as a package and needs additional libraries, which need to be downloaded and installed manually. This led to problems when we installed MiToBo. The problems could be solved with

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the help of the tool's e-mail support. The client-server tools Omero and Bisque also need additional libraries that need to be installed manually.

Second, Table 2 summarizes usability and functionality criteria; all criteria in parentheses in the following sections refer to this table. ImageJ and Fiji fulfill the same criteria with respect to the catalogue and are combined in Table 2.

Documentation (Criteria Group A). Most of the tools provide either a written support or one or another way of electronic support (Handbook, Support). The source code is provided for ImageJ, MiToBo and Omero (Source Code). CellProfiler includes a local description of every parameter. The handbooks from Daime, ColorSeg. and Axio Vision LE are particularly detailed and well written. Icy provides a starter's guide and CellSegmenter good support. The large user community of ImageJ leads to fast support via forum.

Data management (Criteria Group B). The most common nonproprietary file format in fluorescence microscopy is the tagged image file format (TIFF), which stores data and metadata lossless in a single file. Most microscopes support the export of the data to TIFF, storing important meta information such as pixel size and acquisition time in the metadata.

After installation, all tools are able to load and visualize TIFF images. Some are able to load additional proprietary formats: Icy can load the LAF-format and CellProfiler the CVI-format. ImageJ/Fiji, MiToBo, Omero and Bisque integrate the LOCI Bio-formats and are able to import a variety of file formats (Bio-Formats Import). All tools, except Volocity Demo, are able to store the results, and less than half of the tools also provide a file export to a nonproprietary file format (Export). Icy stores segmentation results only with an additional plug-in; Daime uses the proprietary STK-format; ColorSeg. only saves images, not segmentation results; and Omero and Bisque store the results on a server.

Usability (Criteria Group C). Usability refers to an intuitive GUI and menu structure that facilitate working with the tools. ColorSeg. and Volocity Demo are especially user-friendly and in Omero, designed on the basis of various plug-ins, each plug-in itself is intuitively structured (Intuitiveness). Additionally, Volocity Demo and Icy provide user guidance for standard segmentation tasks (Wizard), a feature that is essential for an inexperienced user ('Image processing case study' section). With CellProfiler, only a short orientation phase is necessary before one can start segmenting. Icy provides a starters guide and with Daime one needs a longer orientation phase, but afterwards it is easy to use. The large amount of functionality in ImageJ is mostly organized in the plug-ins menu, which is not logically structured.

Additionally, Figure 1 presents GUIs of some tools. Icy shows a clear GUI, in which icon bars group the methods by topic. The "Detection/Tracking" icon bar collects methods for cell detection and tracking, for example, active contours and thresholding. In CellProfiler, the focus lies on the pipelines concept. The user combines modules collecting locally documented algorithms for one specific processing task to a pipeline. The orientation within the Daime GUI takes some time and although most of the parameters are documented in the handbook, their influence on segmentations remains unclear. ColorSeg. presents the image processing methods in a menu structure and is designed for interactive segmentation. ImageJ arranges the methods in a menu structure and opens each micrograph in a separate window. Fiji's menu structure extends ImageJ's, as it integrates additional methods. The tools in Group IV have a clear GUI; Volocity Demo additionally present the methods in side bars.

Visualization (Criteria Group D). All tools offer basic image viewer functionality, and most can also display histograms (Histogram), but only AxioVision LE let the user adjust brightness and contrast of the images (Adj. Display).

Flexibility (Criteria Group E). ImageJ is a very flexible tool with macros, custom filters and build-in plug-in support. Icy and CellProfiler have plug-in support and Icy additionally supports custom filters (Plug-ins). All tools from Group III and Volocity Demo support the use of macros (Macros).

Segmentation (Criteria Group F). All tools provide a mean for manual, interactive or automated segmentation (Manual, Interactive, Automated). Tools of Group V provide only manual segmentation, whereas tools of Group II offer either interactive or automated segmentations. In CellProfiler, ImageJ, Fiji and Volocity Demo, the user is provided with all three kinds of segmentation methods.

Image processing filters (Criteria Group G). Group I and Groups II–IV partially provide image processing filters such as smoothing or edge enhancement (Preprocessing). All tools other than Omero and Bisque include thresholding (Thresholding). A watershed transform is integrated solely in CellProfiler via seeded watershed; Icy via plug-in; and ImageJ, Fiji and MiToBo via distance transformation of binary images. Icy, Fiji and MiToBo provide level sets (Advanced).

Image processing case study

In the case study, we segmented the four micrographs in Figure 2. Figures 3–6 show the qualitative segmentation results. This results might not be the best, a tool can produce, and is only valid for a qualitative but no quantitative evaluation of usability and functionality.

Fluorescence micrograph 1 (FM1). The test user was able to segment FM1 with all tools that provide thresholding (i.e. Tool

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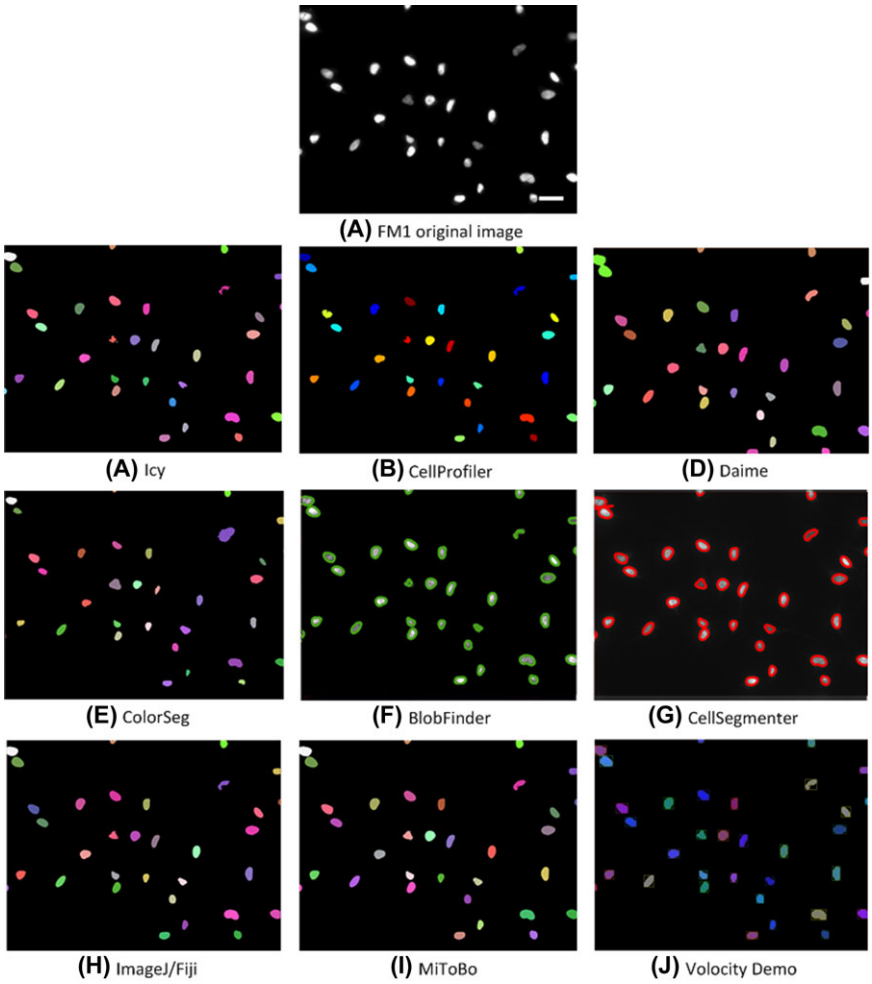

Fig. 3. Segmentation results for FM1 with different tools.

Groups I–III and Volocity Demo) and use different thresholding methods, such as Otsu (CellProfiler), Niblack (MiToBo), local (Fiji), interactive (CellSegmenter) and automatic thresholding (Volocity Demo). The user was able to detect all cells with the tools mentioned above, but the cells differed in size (Fig. 3). This was due to different user-defined thresholds.

Fluorescence micrograph 2 (FM2). In FM2, figure-ground separation could be performed easily and similarly to FM1 with all of the above tools. Cell separation was harder. With ImageJ and Fiji, the distance-based watershed transform produces a cell separation that depends on geometric rather than grey value information. This leads to too many watershed classes and accordingly cells (Fig. 4F). BlobFinder estimates the cell cytoplasm based on a distance transform, which neglects image information. Although the number of classes is more appropriate, in some cases, one cell is falsely separated into two cells (Fig. 4E). In MiToBo, we were not able to use the level set segmentation without a reasonable amount of training with the software, which led to no cell separation and many cells classified as one cell (Fig. 4G, pink cells). In addition, cell separation in Icy left some classes unseparated (Fig. 4B). In Daime, cell separation can be performed with a multiscale morphological approach proposed by Metzler *et al.* (2000). With default parameters, this leads to a coarsely correct but not sufficiently detailed cell separation. With nondefault parameters, further subdivision can be achieved, but the parameter selection is not intuitive and leaves the user to a trial-and-error approach for finding appropriate ones. Also, with a refined separation, some cells should be separated but are not (Fig. 4D; e.g. cyan and blue cells). The best results were achieved with CellProfiler and a seeded watershed approach (Fig. 4C).

Fluorescence micrograph 3 (FM3). FM3 displays contacting HeLa cells with an indistinguishable figure-ground separation, as the contrast between cells and background is less than intracellular contrast. This is reflected by either an oversegmentation of the cells in areas where two cells touch (Fig. 5), or – with tighter thresholds – in an undersegmentation of cytoplasmic border areas, especially in the cell processes. As

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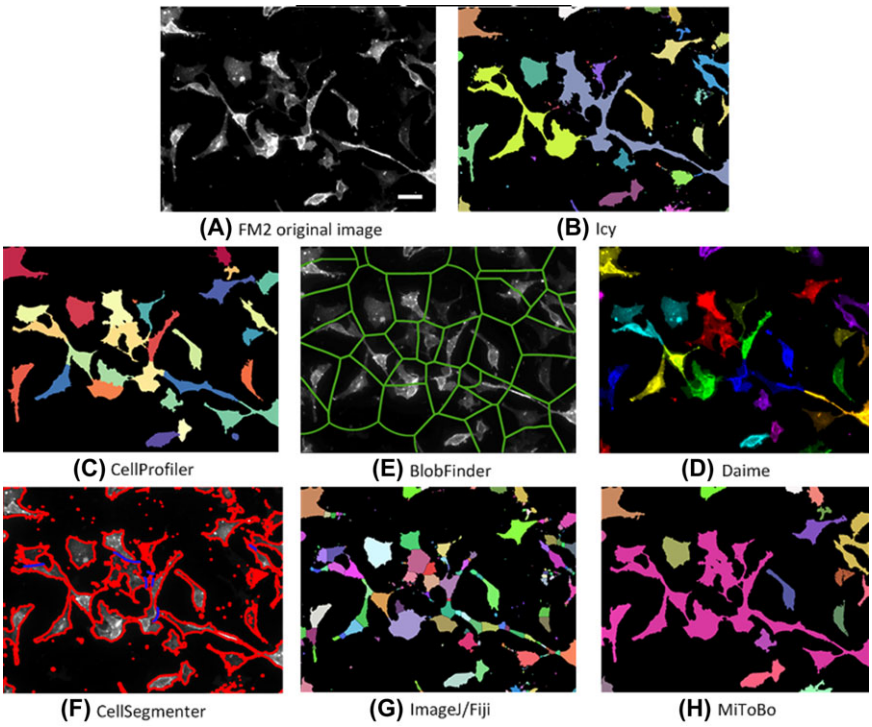

Fig. 4. Segmentation results for FM2 with different tools.

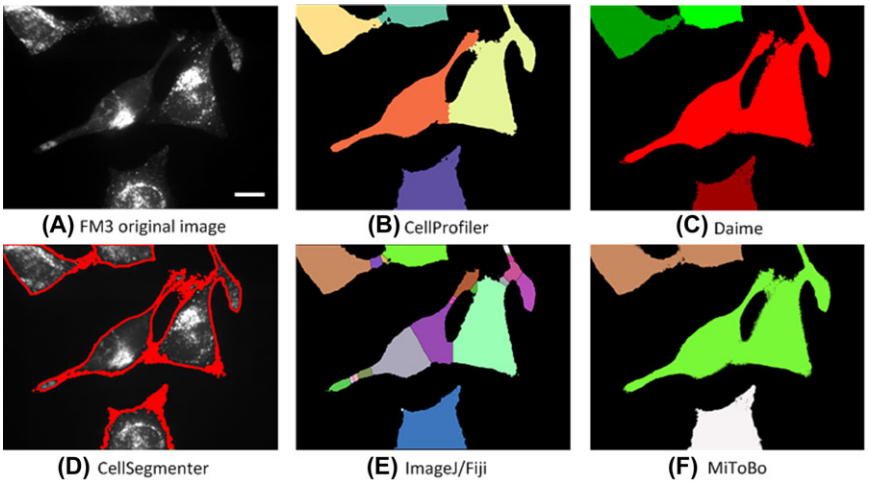

Fig. 5. Segmentation results for FM3 with different tools.

the undersegmentation is difficult to correct, we followed the first approach and needed an additional cell separation step. The results of the cell separations were similar to those for FM2. Only with CellProfiler, we could separate all five cells in FM3 (Fig. 5B). With Daime, only the two cells in the upper left corner could be separated, whereas for the separation of the cells in the middle, no appropriate parameter combination was found (Fig. 5C and previous section). The cell separation with ImageJ and Fiji produced too many classes, which in this case could have been merged manually (Fig. 5E). With tools that provide thresholding, but no further means to separate cells, results similar to those obtained with MiToBo can be achieved (Fig. 5F).

Fluorescence micrograph 4 (FM4). The segmentation of FM4 was mainly a cell separation problem. The figure-ground separation step is a prerequisite for the cell separation step and could again be performed with all tools that include thresholding. The results of the figure-ground separation step can vary due to different thresholds and thresholding

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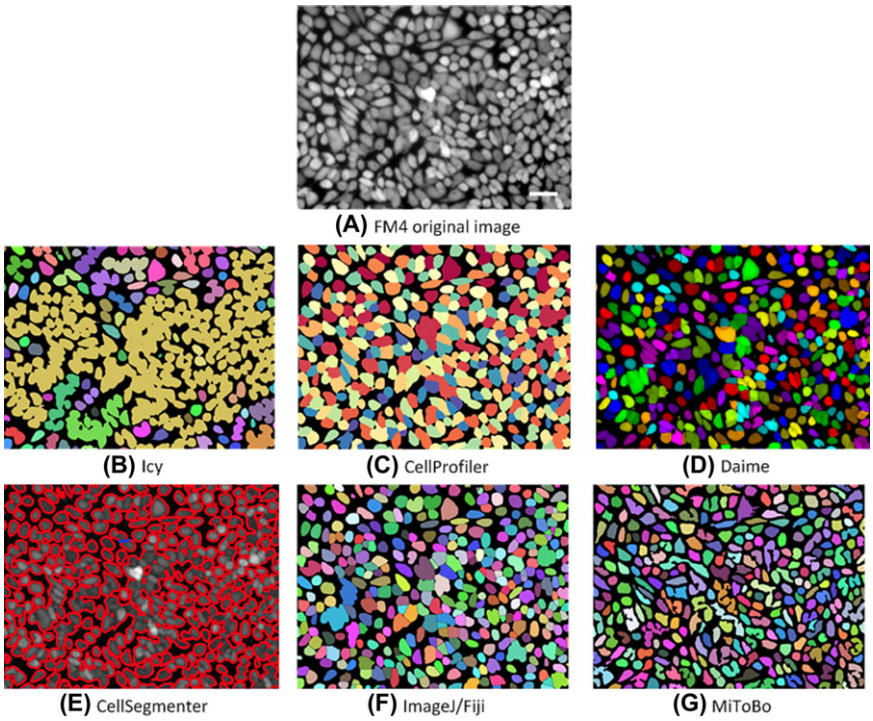

Fig. 6. Segmentation results for FM4 with different tools.

approaches (e.g. Fig. 6C with Otsu threshold and 6G with mean filter and Niblack threshold) and more or fewer cells can be separated even before the cell separation step. Again, CellProfiler's seeded watershed method was able to separate most of the cells correctly (Fig. 6C). As most of them have a similar size, the distance-based watershed approach of ImageJ produced reasonably good results (Fig. 6F), but missed some of the necessary separations. In MiToBo, a combination of a mean filter with Niblack thresholding produced a rather good separation even without a cell separation step (Fig. 6G). With Icy no cell separation was possible after thresholding (Fig. 6B), because the active cell method did not produce results. Although the cell separation method in Daime separates some but not all of the cells, some cells were not correctly separated (Fig. 6D).

Usability-functionality graph

Figure 7 shows the degree of functionality versus the degree of usability of the tools. The scatter plot (Fig. 7) displays the combined results according to our criteria catalogue (Table 2) and the user case study. Usability includes Criteria Groups A–D on the vertical axis and functionality Criteria Groups E–G on the horizontal axis. We weighed some of the criteria twice: Support solves some problems with a tool; Intuitiveness shortens the user's orientation phase with the GUI; Wizards help to shorten the training phase; Interactive and Automated methods process large amounts of image data; Thresholding is widely used for figure-ground separation; and Advanced methods are a prerequisite for automated cell separation.

Discussion

In the next section, we discuss how the results of the criteria catalogue, the image processing case study and the usability-functionality graph affect usage of the studied tools.

Criteria catalogue

Structural requirements for all tools are in the same range and are met by standard personal computers with Windows XP or 7; for other operating systems, the tools from Groups II and IV are not available. For most tools, except MiToBo, installation is easy. For MiToBo, an installation tutorial exists. CellSegmenter is only installable with a valid Matlab license and Tool Group III with Java. Criteria Groups are discussed in the following sections.

Documentation (Criteria Group A). Tool documentation is necessary to shorten a user's training period with a tool. In particular, complex tools require extensive documentation that help the user find and use existing methods. ImageJ/Fiji integrate extensive functionality, but lack documentation, which results in a longer training period. Compared to ImageJ/Fiji, CellProfiler integrates the same level of functionality, but provides local parameter documentation, which reduces the training period. BlobFinder integrates less

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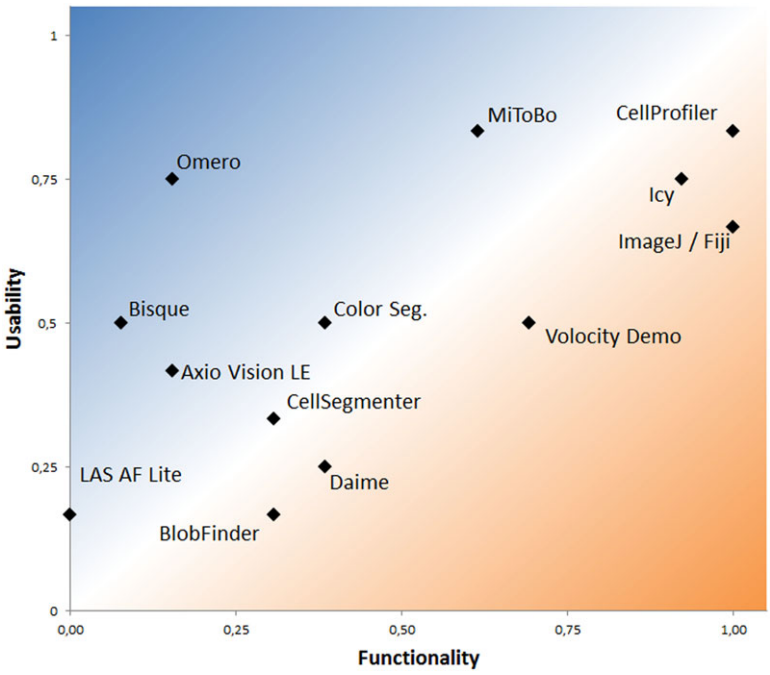

Fig. 7. Usability-functionality graph of the examined image processing tools.

but specialized functionality. This tool therefore has a low complexity and does not need documentation to shorten the user's training period. We suggest using tools for which documentation matches functionality.

Data management (Criteria Group B). Tools that only provide data import and export to proprietary files force the user to perform additional data conversions to, for example, the TIFF format. Although there are tools available to perform almost all kinds of fluorescence micrograph conversions, the user needs additional training and time to perform the conversion step. A conversion might also lead to data loss, which directly influences the segmentation results. We recommend the tools (from Groups III or IV) that integrate LOCI BioFormats (Import, Table 2), or fit in an existing tool chain.

Usability (Criteria Group C). Tools with intuitiveness (GUI and menu structures) allow for a reduced training period, reduced time to orient in the tool and reduced search time for specific functionality. This criterion is highly dependent on the field of expertise of the user. In a tool that is structured for a life scientist, categories reflect the user's mental categories. Therefore, life science users would prefer tools with a problem-oriented structure to tools with an image-processing-method-oriented structure and should try to avoid tools with no or only a coarse structure. Wizards restrict a method's complexity and the user's flexibility. Both are necessary to facilitate the usage of complex methods in tools for life scientists.

Visualization (Criteria Group D). A low signal to noise ratio and a low contrast of cells outside the focus plane of the microscope characterize fluorescence cell micrographs. Thus, the adjustment of brightness and contrast in the software helps the user locate and characterize all cells in a fluorescence micrograph. We recommend either to use a tool with the Adj. Display criteria or to adjust brightness and contrast with an image processing filter (Preprocessing, Criteria Group G). Low contrast micrographs also need to be adjusted using Daime, ColorSeg., BlobFinder and CellSegmenter to analyze them. The histogram of a micrograph helps the user with interactive thresholding (Thresholding, Criteria Group G).

Flexibility (Criteria Group E). Users with a large number of micrographs in one study should choose tools with a macro-mode (e.g. CellProfiler, Tool Group III and Volocity Demo). With plug-in support, a user can extend a tool he is already familiar with and use additional functionality with the user interaction concept he is used to. The plug-in criteria are therefore important for the user who needs to handle different image processing tasks with one tool.

Segmentation (Criteria Group F). Studies that analyze only a few micrographs and cells are well suited for manual or interactive methods. Studies with large numbers of micrographs and cells require automated methods in combination with macros (Criteria Group E). Automated methods not only reduce user interaction but also increase reproducibility of the results.

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Image processing filters (Criteria Group G). Preprocessing is necessary to enhance image quality, either for supporting image processing methods or to improve visibility of cells (to compensate for a missing Adj. Display criteria). Thresholding methods are most important when performing figure-ground separation in fluorescence micrographs, because they are easy to use and adjust. The threshold criteria are crucial. If the user is not willing or able to evaluate a study manually, we do not recommend tools without any means for thresholding (AxioVision LE, Tool Group V). Cell separation can also be performed manually, or with advanced methods. Advanced methods are included in Icy, CellProfiler and Group III. However, they are often hard to use and adjust (see discussion in 'Image processing case study' section).

Image processing case study

The most important result of the case study is that the interaction with a tool is crucial for fluorescence image analysis. The following points are necessary prerequisites for a successful image analysis: The functionality needs to be available in the tool and the user needs to be able to find and use the functionality. These points are discussed in the following sections concerning the interactive and automated segmentation methods, since their results are reproducible in contrast to manual segmentation.

The segmentation results show that most software tools provide one or the other thresholding method and can be used for figure-ground separation in all of the study's fluorescence images. The cell separation step, which is often necessary, remains the challenging one. Although in some tools, advanced methods are integrated (Table 2), they are either difficult to use or are not able to separate cells appropriately. For example, for Level Sets in Icy, Fiji and MiToBo, an initialization and parameter set is difficult to configure. If Daime's morphological cell separation method does not work with default parameters, it needs to be parametrized with appropriate values. ImageJ's and BlobFinder's distance-based cell separation methods produce suboptimal results. The most promising approach is the seeded watershed approach in CellProfiler. It is integrated in the IdentifySecondaryObjects filter, together with figure-ground separation, and needs no parameter.

Additional to the availability of the appropriate methods, the user interface, as the link between user and the tools, becomes the crucial point for fluorescence image segmentation. The user needs to know what methods are required and find them in the tool. Here, the expertise of the user, either as a life scientist or as an image processing expert, makes a huge difference on how user friendly a tool is perceived. Although life scientists know **what** they want to do, image processing experts know **how** to do it. This means the menu structure for life scientists needs to be sorted into possible applications in life science and for image processing experts into image processing categories. In addition, the user needs guidance in parameter selection for available methods. For this, good default parameters and a reduction in the number of necessary parameters are desirable. A reduction of parameters can be achieved with, for example, supervised learning (Held *et al.*, 2011). A sound and easy to find description of the parameters, their value range and how the user needs to adjust them needs to be included. Also, it is beneficial when parameter changes lead directly to changes in the resulting image. This gives the user feedback in the time-consuming, trial-and-error process of finding the right set of parameters.

Many image processing research tools do not provide a user interface tailored to life science or sufficient parameter descriptions. This means that most users will not be able to use the full functional scope of such research tools. This is especially prominent in ImageJ, which has a huge functional scope, but most of the functionality is hidden behind a confusingly unsorted menu structure. For application in daily routines tools with a more restricted but specific functional scope are easier to handle and need less user orientation within the tool. Volocity Demo and CellSegmenter are examples of small tools with a clear menu structure. The user's necessary orientation phase can further be shortened with user guidance. A good example of a user-friendly tool, which avoids image processing terminologies, has a clear menu structure and provides useful feedback to the user, is the CellProfiler.

Mühler *et al.* (2008) provided guidelines for surgical planning tools. Their two most important findings also apply to fluorescence image analysis tools:

(1) Less is More.
(2) Less Flexibility, More Guidance.

Nevertheless, no tool prevents the user from developing an appropriate image processing pipeline. This may be a feasible task for some experiments, but can also require expert knowledge.

Usability-functionality graph

Figure 7 displays the balance of usability versus functionality in the tools. Most tools are well balanced and located in proximity to the diagonal bisector. In tools in the upper left half of the graph, usability exceeds functionality, whereas in tools in the lower right half, functionality exceeds usability. The left half of the graph contains tools with a restricted functional scope, while the right half with extensive functionality. The lower half collects tools with lower usability, and the upper half tools with higher usability.

Tools in the lower right half, Omero, Bisque and AxioVision, offer a lot of support and documentation but only manual annotation, which renders them unusable for datasets with many cells to segment.

CellProfiler offers the most functionality combined with the best usability result, but lies below the bisector. Users have a

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lot of flexibility during the evaluation process, but they are also challenged to select the appropriate algorithms, which may also require image processing know-how. The general purpose tools such as ImageJ/Fiji and Icy also include more functionality but lack more usability due to less user guidance compared to the CellProfiler. Therefore, greater image processing knowledge is recommended for users. MiToBo, with its well-structured menu, contains less functionality than ImageJ and is relatively better to use. Specialized tools such as ColorSeg., CellSegmenter, Daime and BlobFinder are designed for special segmentation tasks with one single algorithm. Their usage requires no or only little image processing know-how and only a short orientation phase. They are suitable if users' problems are solved by the chosen tool.

The tools in Group IV are versions with limited functionality. This had a strong influence on their test performance. The only restriction in Volocity Demo is that the segmentation results cannot be stored. This means that the users can only evaluate the images within the software, but the results cannot be published.

There are three questions a life scientist needs to ask, when he wants to select an image processing tool for a given study: 1) How complex is my problem? 2) How often do I need to perform image processing? 3) Am I willing to spend time in training? If the complexity of the problem is high or the life scientist will deal with image processing tasks often, training time with a more complex software will pay out. For a life scientist, who infrequently deals with image processing, the tools with the highest usability in the graph, which provides an adequate functionality for the given problem, will suit best.

In general, we suggest using the most specialized tool that solves a specific problem. If a problem is complicated, or a user wants to use a tool for different fluorescence micrograph studies with different problems, the longer training phase of a complex tool will pay out. If a study requires the analysis of many cells, at the very least an interactive or a better automated method is necessary. If the study also includes a large number of micrographs, then automated methods with macros should be used.

Conclusion

We have presented a review of 15 free software tools for cell fluorescence micrograph evaluation. The tools were compared with respect to a criteria catalogue containing usability and functionality criteria. An image processing case study revealed the importance of user guidance within the tools, especially for complex image processing tasks. We discussed how the criteria catalogue can be used to select a tool that best matches the requirements of a fluorescence micrograph study. There is no one-and-only tool for all possible users or use cases, but there are tools, for better or worse, bridging the semantic gap between image processing methods and life scientists.

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