Biological imaging software tools

Kevin W Eliceiri[1], Michael R Berthold[2], Ilya G Goldberg[3], Luis Ibáñez[4], B S Manjunath[5], Maryann E Martone[6], Robert F Murphy[7], Hanchuan Peng[8], Anne L Plant[9], Badrinath Roysam[10], Nico Stuurman[11], Jason R Swedlow[12], Pavel Tomancak[13] & Anne E Carpenter[14]

Few technologies are more widespread in modern biological laboratories than imaging. Recent advances in optical technologies and instrumentation are providing hitherto unimagined capabilities. Almost all these advances have required the development of software to enable the acquisition, management, analysis and visualization of the imaging data. We review each computational step that biologists encounter when dealing with digital images, the inherent challenges and the overall status of available software for bioimage informatics, focusing on open-source options.

The last 20 years have seen great advances in optical imaging with the introduction of the ability to monitor biological phenomena with unprecedented resolution, specificity, dimensionality, complexity and scale, all while maintaining viability and biological relevance. These imaging modalities, which are increasingly multiparametric, rely heavily on computational approaches. In fact, in many cases the computational technology is just as important as the optics, not just for the digital capture that all systems now use but in many cases also for visualizing and properly interpreting the data. In the quest for breakthroughs, biologists are often confronted with the challenge of processing digital data of increasing complexity and richness, which demands an informatics infrastructure with tools to collect, store, manipulate, analyze, interpret and visualize vast amounts of imaging data in a reproducible manner with the flexibility to refine aspects of their experimental and imaging techniques in a tight iterative loop.

Despite this great need, the bioimage informatics field is still a fairly nascent community compared to the more established hardware-development side of the optical microscopy community. But with the increased mainstream adoption of advanced optical-imaging approaches by biologists and the commitment by funding agencies to prioritize bioimage informatics, there has been a great increase in the number of bioimage informatics[1] tools over the last 5 years.

For this Review, representative members of the bioimage informatics community collaborated to review each computational step that biologists encounter when dealing with digital images (**Fig. 1**), the challenges in that domain and the overall status of available software for bioimage informatics, focusing on open-source options. Our goal is to provide an overview of how open-source imaging software can be used to provide an end-to-end laboratory solution from data acquisition and storage to data analysis and mining.

IMAGE ACQUISITION

In biological laboratories images are usually acquired by measuring photon flux in parallel (using a camera)

[1]Laboratory for Optical and Computational Instrumentation, University of Wisconsin at Madison, Madison, Wisconsin, USA. [2]Department of Computer and Information Science, Universität Konstanz, Konstanz, Germany. [3]National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA. [4]Kitware Inc., New York, New York, USA. [5]Department of Electrical and Computer Engineering, Center for Bio-image Informatics, University of California Santa Barbara, Santa Barbara, California, USA. [6]National Center for Microscopy and Imaging Research, University of California San Diego, La Jolla, California, USA. [7]Lane Center for Computational Biology, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA. [8]Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, Virginia, USA. [9]Biochemical Science Division, National Institute of Standards and Technology, Gaithersburg, Maryland, USA. [10]Department of Electrical and Computer Engineering, University of Houston, Houston, Texas, USA. [11]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California, USA. [12]Wellcome Trust Centre for Gene Regulation and Expression, University of Dundee, Dundee, UK. [13]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. [14]Imaging Platform, Broad Institute of Harvard and the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. Correspondence should be addressed to K.W.E. (eliceiri@wisc.edu) or A.E.C. (anne@broadinstitute.org).

PUBLISHED ONLINE 28 JUNE 2012; CORRECTED AFTER PRINT 20 JULY 2012; CORRECTED AFTER PRINT 29 AUGUST 2012; DOI:10.1038/NMETH.2084

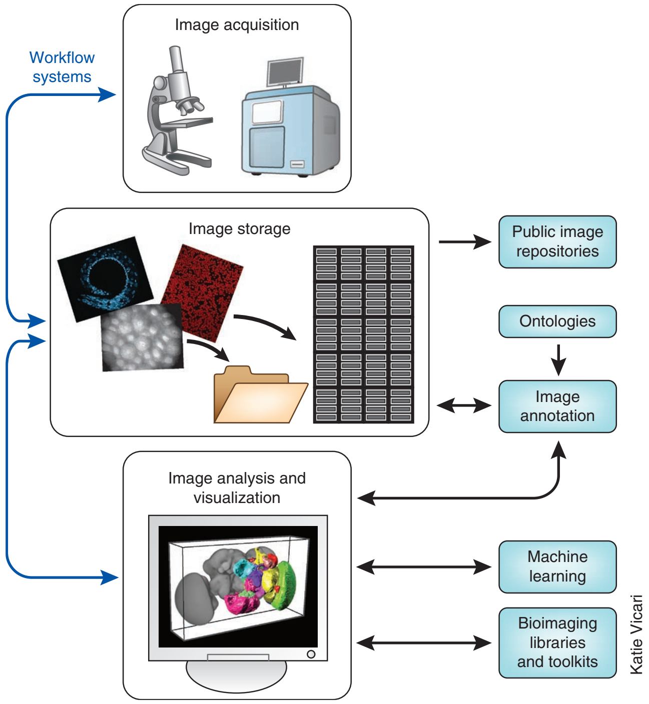


Figure 1 | Overview of imaging workflow. Modern bioimaging requires the use of software tools for most stages of the workflow.

or sequentially (using a point detector and equipment that scans the area of interest). Although capturing an image from a camera into the computer is straightforward, in most cases image acquisition needs to be tightly synchronized with other computer-controllable equipment such as shutters, filter wheels, *x*-*y* stages, *z*-axis focus drives and autofocus mechanisms (implemented in software or hardware). This automation is necessary to gather desired multiparametric information from a sample or to allow the unattended acquisition of large numbers of images in time-lapse series, *z*-stacks, multiple spatial locations in a large sample or multiple samples in a large-scale experiment. Image-acquisition software is therefore needed to communicate with these various components and coordinate their actions such that the hardware functions as quickly and flawlessly as possible while permitting the researcher to easily design and execute the desired sequence of acquisition events (**Fig. 2**).

Confocal microscope systems that require computer control to acquire an image are almost always bundled with control software, whereas typical wide-field microscope systems are not, requiring the researcher to choose between several different independent software packages for microscope control and automation. But not all software packages support all microscopy-related hardware. Because the cost to write code to support hardware is high, the choice of which hardware a given software package will support is typically driven by commercial interests, and support for a hardware component can rarely be added by third parties. Hence, software compatibility with hardware is an essential consideration when planning a new microscope system.

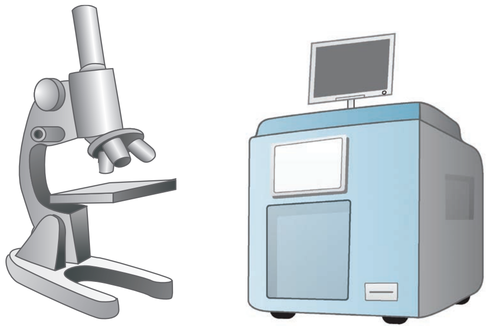
Several commercial software packages combine image acquisition and analysis. These include Metamorph (Molecular Devices), SlideBook (3i), Image-Pro (MediaCybernetics) and Volocity (Perkin-Elmer). Most of these software packages were initially developed in individual research laboratories and were commercialized later. In addition, each of the major microscope companies have their own software packages, such as AxioVision (Zeiss), NIS-Elements (Nikon) and cellSens (Olympus).

The obvious advantage of commercial image-acquisition packages is that they provide a turnkey solution to all 'standard' image-analysis strategies (acquiring individual images, taking time-lapse series, collecting three-dimensional (3D) stacks at multiple *x*-*y* positions and so on). Moreover, when such packages are purchased as part of an imaging system, there is less danger they will not be compatible with the system's configuration. It is virtually impossible, however, for individual researchers to substantially extend any of these software packages or make substantial custom hardware changes to the imaging system. This reduces the rate at which new imaging techniques can be transferred to laboratories outside that of the inventor.

Researchers that have nonstandard or frequently changing needs and equipment must often write their own code. Software development is facilitated by toolkit environments such as LabView (National Instruments) and Matlab (Mathworks), which provide interfaces to a subset of available equipment and can be used to create a graphical user interface. Developing new imaging technologies necessitates writing instrument-control code, as the needs for these new techniques simply could not be anticipated in existing software packages. Examples of developments enabled by software written in research laboratories include structured-illumination microscopy[2], super-resolution microscopy[3–5] and Bessel-beam microscopy[6]. Although toolkit environments provide high flexibility and are well suited for tools intended for the group that wrote them, they are less appropriate for distributing these tools to others primarily because the infrastructure back-ends are costly and the distribution channels are not sufficiently developed.

Two open-source software projects, µManager and ScanImage, whose development is driven by researchers, are intended to provide tools with more flexibility than commercial tools and greater ease of use than the toolkit environments in terms of microscope control. µManager mainly targets camera-based imaging, although it is also used with scanning systems[7]. It includes an easy-to-use interface that runs as an ImageJ plug-in and enables researchers to design and execute common microscopy functions as well as customized image-acquisition routines. The solutions can be easily distributed as scripts or plug-ins. µManager's hardware abstraction layer can also be used without its ImageJ user interface in environments such as Icy, Matlab, LabView and Python, facilitating the development and transfer of software for new imaging approaches. The software framework enables any entity, academic or commercial, to write and contribute its own device adapters for hardware components. µManager provides full control of the components of the light microscope, such as cameras, stages and filter wheels. The program can be used to collect multichannel data over space and time, such as tracking fluorescently tagged cell fusion events in live cells in a multiwell plate overnight[8].

Another open-source package, ScanImage, provides a software framework to control laser-scanning microscopes and is used extensively for two-photon excitation microscopy[9]. It implements most standard modes of image acquisition and basic automation, and supports continuous image acquisition synchronized



- Manual or automated acquisition
- Single time point or time series
- Single focal plane or three-dimensional stack
- Single channel, multiple channels or hyperspectral
- Acquisition protocol is predefined or determined on the fly based on analysis of image data

Katie Vicari

Figure 2 | Image acquisition spans a range of complexity and variation.

to behavioral or physiological data, which is particularly useful for imaging in intact animals. The software framework is object-oriented and event-driven to promote extensibility, online analysis and plug-in development. ScanImage complements μManager in that it can control laser-scanning microscopes, such as in-house–built confocal systems, and allow for complex recordings where high signal-to-noise ratio is needed, such as tracking axon signaling in neuron cultures.

These projects exemplify certain benefits that can arise organically from open-source bioimaging software. Open-source software in this domain aids the scientific community by facilitating the rapid dissemination of new optical techniques from labs focused on instrumentation development, without requiring them to also run major software efforts. Furthermore, μManager now supports so many different manufacturers' cameras that its interface has essentially become a *de facto* standard for controlling scientific-grade cameras, providing benefits to the scientific community and to commercial vendors.

IMAGE STORAGE

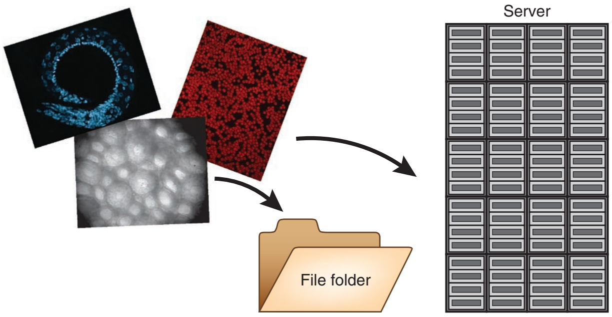
The vast increase in data volume and the complexity of bioimaging experiments and acquisition protocols has rapidly made a paper lab notebook an unsuitable solution for keeping track of information about imaging experiments. In fact, finding, viewing or analyzing the data with everyday computer software is typically infeasible. In many laboratories, tens to hundreds of gigabytes of image data per day are routinely generated. New sophisticated automation techniques[10] promise to increase and accelerate this trend. 'Enterprise-level' data generation must therefore be matched with software applications that can properly manage, view, share, process and analyze these data (**Fig. 3**).

In the last 10 years several groups have developed applications for managing large collections of scientific images. These are often referred to as 'image databases'. These image databases provide integrated platforms to organize, search, process, analyze, share and visualize data associated with biological experiments, including both images and image metadata. Such systems for image storage and retrieval are quite dependent on suitable annotation of images, both in terms of describing the experimental details used to create the image as well as the automatic or human interpretation of the content of the image (**Box 1**). The usefulness of image annotation is greatly enhanced by ontologies that formalize the names and relationships among image metadata (**Box 2**).

Two examples of open-source bioimaging database projects are the Open Microscopy Environment's Remote Objects (OMERO) platform[11] and the Bio-Image Semantic Query User Environment (BISQUE) project[12]. Both are web-based open-source projects that allow users to extend both metadata and workflow models for their individual applications. Both also invoke the idea of 'remote access': a scientist can work with his or her data using a standard internet connection. This type of technology is referred to as a 'client-server architecture' where a server application holds and manages the data and delivers a view of the images, metadata, annotations and analytic output in a client such as a web browser. This architecture provides a framework where researchers use tools hosted on a server to process and analyze large image data collections, taking advantage of the increasing power of computational resources in clusters and in the cloud.

Image databases can be searched using annotations, and relevant images can also be found by content-based image retrieval, also known as 'query by image content'. This involves searching for images that are similar to a query by some measure of distance using features or model parameters (we discuss these below). As an example, the open-source software OMERO.searcher provides this capability, building on the Feedback Adaptive Loop for Content-Based Retrieval (FALCON) algorithm[13] used in the Protein Subcellular Location Image Database (PSLID).

BISQUE and OMERO are just two examples of a growing ecology of software systems to help scientists manage image collections. Commercial solutions such as the Columbus Image Data Management system (PerkinElmer) based on OMERO, and the PCI Image Database (Quartz), are also available. The BISQUE platform is integrated with the iPlant cyberinfrastructure to provide a scalable image management and analysis platform for plant biologists[14]. Given the vast variety of applications and contexts for bioimaging experiments, it is nearly impossible to create



- Image transfer and storage: manual or automatic
- Images stored on a local hard drive or a remote server
- Files deposited into folders, an instrument-specific database or a general image file management system
- Single copy of data or periodic backups
- Image metadata hand-written in lab notebook, stored in an electronic notebook or stored in an image file management system

Katie Vicari

Figure 3 | Options for image storage vary in complexity and size.

BOX 1 IMAGE ANNOTATION

To be analyzed, retrieved, visualized and/or shared, biological images require annotation, the process of associating images with metadata (information about the images, including information about how the images and the samples were created as well as information about the content of the image itself). Whether using formal ontologies (**Box 3**) or informal means of annotation, describing images in a systematic and machine-computable manner is often critical for making full use of them, either owing to the scale of the bioimaging experiment or the reuse of image data for a new purpose. Unlike some other readouts, biological images can contain a vast amount of information that is often not fully extracted in the initial analysis.

Despite its recognized value, image metadata capture and annotation has not yet become widespread for bioimaging experiments[57], though efforts are underway in certain areas. A major challenge is that capturing all types of metadata for a bioimaging experiment is currently time-consuming and impractical. This is in part because the inherent amount of annotation required for all conceivable downstream uses of imaging data is overwhelming, causing most researchers to record just the information necessary for their own purposes. The challenge also lies in the lack of user-friendly annotation tools to entice biologists to regularly record metadata.

Some tools are in development to ease the annotation process for researchers. For image acquisition, microscopes usually automatically store information about image acquisition in the header of the resulting image files. Unfortunately, these metadata and even the pixel data are often stored in a proprietary form. The Open Microscopy Environment has two solutions to help address the challenge of proprietary image formats and allow for easy and robust harvesting of acquisition metadata. The Bio-Formats project[57] is a library used by many open-source and commercial imaging software tools that allows for the full parsing of more than 120 proprietary image formats and the accurate conversion of the proprietary metadata to the OME-XML data model. The OME-TIFF project is a container format for the OME-XML data model and serves as the main export format of Bio-Formats. OME-TIFF is therefore a practical choice for software tools for which the aim is to record metadata in an open image format. The OMERO data system of the Open Microscopy Environment also offers image annotation with current support for text and graphical annotation of regions of interest for images stored in the database. The Cell-Centered Database Web Image Browser tool is in development to enable manual annotation of the organism type and anatomical region where the image or volume is found (http://openccdb.org/software/index.shtm#wib/). To encourage biologists to record experimental details leading to the capture of images, especially for complex experiments, ProtocolNavigator captures protocols as a visual map (http://vizbi.org/Posters/2011/D06/).

Although images must often be annotated through manual curation, approaches for automated annotation of image content are in development and are especially needed given the sheer number and volume of images that are now routinely produced. These systems, whether providing automatic collection of metadata at acquisition or manual annotation after acquisition, are in the early stages of implementation and adoption. As the field increases its demand for quantitative analysis and robust curation and sharing of the image data, the need for full ontologies and annotations will increase. Annotation is needed at every stage of the biological imaging workflow, but systems covering both manual and automated annotation of the full workflow still need to be developed and implemented.

a universal solution for all scientists' needs. Rather, scientific image databases must be flexible and able to integrate applications and functionality demanded by the scientific application even if unanticipated by the database developers. This is why BISQUE and OMERO provide adaptable frameworks and not complete, monolithic applications. BISQUE and OMERO leverage the latest enterprise database and file server technology and couple that with the unique requirements of the imaging community. Together they represent a class of imaging database solutions that can be deployed in an individual lab, a network of labs or publicly available repositories (**Box 3**).

This flexibility stems in part from the use of open-source development practices that allow the community to review, contribute and participate in the development project and also facilitates connections to other open-source bioimaging software. For example, both OMERO and BISQUE can work with CellProfiler and ImageJ, and implementations exist for interfacing with Matlab (MathWorks). Further, BISQUE and OMERO share data models. BISQUE supports the OME XML data model and uses the OME Bio-Formats to import proprietary microscopy image data. Given the rapid development of analysis and visualization tools for imaging, this type of collaborative use of code and integration promises to provide very powerful tools for biologists now and in the near future.

IMAGE ANALYSIS AND VISUALIZATION

Although there is no denying the importance of every step in the bioimaging pipeline, the heart of bioimage informatics is, of course, the images themselves and methods for their analysis and visualization. These two processes are inextricably linked, and image data derived from different imaging modalities, applications and experimental designs require a rich diversity of ever-evolving tools and techniques to extract biologically meaningful quantitative data from myriad types of microscopy images. We provide a high-level overview of the tools available and encourage readers to explore online resources and cited papers for detailed information.

Image analysis

Biologists are increasingly interested in using image analysis to convert microscopy images into quantitative data[15,16]. In particular, image analysis is a necessary step for experiments in which hundreds or thousands of images are collected by automated

BOX 2 ONTOLOGIES

Annotation of images, to be useful for image retrieval and analysis across experiments and laboratories, is greatly enhanced through the use of ontologies. Ontologies are formal expressions of human knowledge about a domain in machine-readable form[58]. Ontologies enable the consistent description of image metadata, such as the type of microscopy or stains used and the cell or tissue type imaged. They can also enable consistent description of visual annotations by defining a set of classes (such as cell or organelle) and the relationships between them (an organelle is part of a cell).

There are a number of valuable benefits to image annotation with ontologies. First, at the most basic level, ontologies provide an effective and flexible means to apply controlled vocabularies to annotation. This allows multiple terms (synonyms, plurals and lexical variants) to be resolved as describing the same item and disambiguates multiple items being described using the same single term (for example, the nucleus of a cell, the nucleus of the brain and the nucleus of an atom). Second, ontologies are computable. A well-constructed ontology names each class via a unique identifier, ideally structured in the form of a uniform resource identifier, which serves as an easily computable 'handle'. Third, when constructed using formal logical languages such as OWL (web ontology language), ontologies can perform categorization through a reasoning process similar to that of a human. For example, ontologies enable the generation of new hierarchies based on rules and axioms (for example, a Purkinje cell is a type of GABAergic neuron because it is both a type of neuron and uses GABA as a neurotransmitter). By expressing image annotation in machine-computable form as a formal ontology, human knowledge can be brought to bear on effective search and interpretation of image data, especially across multiple disciplines, scales and modalities.

Ontologies and ontology-based services can be built into databases. Tools exist for certain metadata relevant to bioimaging experiments and are in development for others, through efforts such as the Open Biological Ontologies project, the US National Center for Biomedical Ontologies Bioportal and the Neuroscience Information Framework. Ontologies now cover major biological entities, such as cell components (Gene Ontology), chemicals of biological interest (Chebi), anatomical entities (Foundational Model of Anatomy) and cell types (Cell Ontology). Other ontologies cover entities describing data acquisition (OME-XML), experimental techniques and protocols (Ontology of Biomedical Investigation) and data elements (Information Artifact Ontology). However, the process of formal ontology construction is slow and may not keep pace with rapidly evolving imaging technologies. Systems have also been proposed by which highly granular vocabulary terms can be developed and evolved locally into an ontology by the experimentalist, with guidance provided that encourages concordance with existing ontologies (ref. 59 and http://xpdb.nist.gov/bioroot/bioroot.pl/).

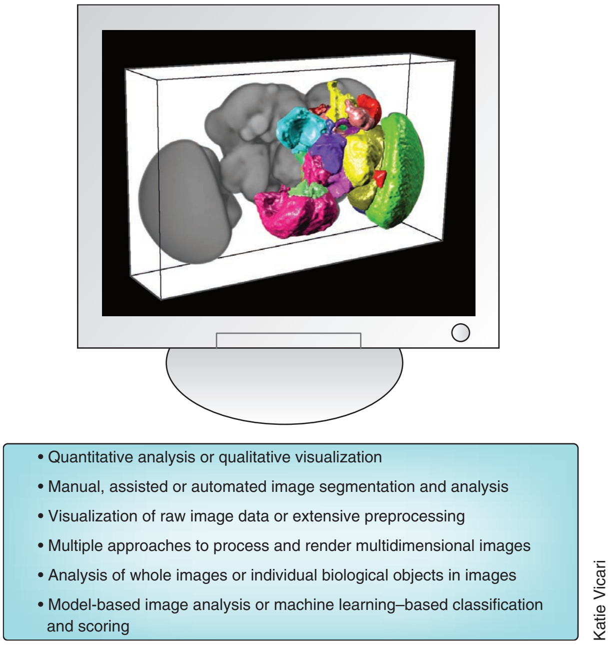
microscopy, whether for screening multiple samples, collecting time or *z*-series data, or other technologies that generate vast volumes of image data. In addition to image analysis in a high-throughput context, image processing is important for many biological studies: for example, quantifying the amount and localization of a signaling protein, measuring changes in structures over time, tracking invading cancer cells or looking at nonspatial data such as fluorescence-lifetime data[17]. Image analysis can help ensure that results are accurate, objective and reproducible (**Fig. 4**).

A vast number of image-analysis algorithms and software packages have been developed for biological applications, especially in the past decade. The software packages differ in their intended application areas, usability, openness of the source code and cost. Whereas in the past proprietary file formats often necessitated the use of only the commercial software bundled with the microscopes, third-party software programs can now be used for most images generated by most instruments, either directly or through file format readers. Given the number of open-source and commercial solutions, it is often difficult to choose which tool is appropriate for a given task.

Below we highlight some of the more prominent and widely used examples that have been proven useful for light microscopy across

BOX 3 PUBLIC IMAGE REPOSITORIES

Public bioimage data repositories have been available for several years, and the number of them is growing[60]. Some repositories are geared to education and outreach, such as the The Cell: An Image Library (http://www.cellimagelibrary.org/) and The Cell Centered Database (http://ccdb.ucsd.edu/), which merged in 2012. Others gather sets of images that accompany published papers, such as the Journal of Cell Biology (JCB) DataViewer (http://jcb-dataviewer.rupress.org/), which provides full access to original multidimensional image data associated with articles published in the *Journal of Cell Biology*. Still others are more focused on providing research resources, often focused on particular domains. These include the Human Protein Atlas (http://proteinatlas.org/), the Allen Brain Atlas (http://www.brain-map.org/), the e-Mouse Atlas Project (http://www.emouseatlas.org/), the Protein Subcellular Location Image Database (http://pslid.org/), the Worm Atlas (http://www.wormatlas.org/), *Drosophila* Image Repositories (http://flybase.org/) and LOCATE (http://locate.imb.uq.edu.au/). Methods for searching, analyzing and distributing images from these databases are still developing. Using standard methods of tracking access and downloads will help identify which data are most valuable for the community and which applications and functionality are most scientifically useful.



- Quantitative analysis or qualitative visualization
- Manual, assisted or automated image segmentation and analysis
- Visualization of raw image data or extensive preprocessing
- Multiple approaches to process and render multidimensional images
- Analysis of whole images or individual biological objects in images
- Model-based image analysis or machine learning–based classification and scoring

Katie Vicari

Figure 4 | Image analysis and visualization span a range of complexity and variation.

many biomedical research areas. We focus on image-analysis and visualization tools that support interoperability with each other and with software for other steps of the bioimaging workflow (such as data acquisition and storage).

Niche image-analysis tools. Most image-analysis software packages developed in academia are written to accomplish very specific tasks relevant to a research problem at hand. Software exists that is designed solely for particular cell types (especially neurons), organisms, assay readouts and imaging modalities. There are literally hundreds of such tools, both active and discontinued. One more prominent example is the fluorescence association rules for multidimensional insight (FARSIGHT) toolkit, which grew out of the need to map the glio-vascular substrate of brain tissue surrounding neuroprosthetic devices. Owing to the number and variety of such tools, it is sometimes appropriate to find this software via web search engines or through online listings for certain biological specialties, such as the Neuroimaging Tools and Resource Clearinghouse (NITRC), which focuses on image analysis for neuroscience (http://nitrc.org/), or the US National Institutes of Health–funded Computer Integrated Systems for Microscopy and Manipulation (http://cismm.cs.unc.edu/resources/external-links/).

Generalist image-analysis tools. The second category of image-analysis software packages is those that can address more general problems. These software packages are typically modular and thus offer greater flexibility to multiple applications. Some commercial tools in this category include MetaMorph, Amira (Visage Imaging), Volocity, Imaris (Bitplane Scientific Software), NIS-Elements, SlideBook, ImagePro Plus (Media Cybernetics) and ZEN (Zeiss); these are often offered by microscopy companies and sold together with imaging instrumentation. There are many open-source image-analysis solutions originally developed to solve the needs of a particular community but later used or expanded to other purposes, such as BioImageXD[18], Icy[19], Fiji[20], Vaa3D (3D visualization–assisted analysis)[21], CellProfiler[22], 3D Slicer (S. Pieper, B. Lorensen, W. Schroeder and R. Kikinis; *Proceedings of the 3rd IEEE International Symposium on Biomedical Imaging: From Nano to Macro*, vol. 1; 2006), Image Slicer, Reconstruct[23], FluoRender (Y. Wan, H. Otsuna, C.-B. Chien and C. Hansen in *Proceedings of the IEEE Pacific Visualization Symposium*, 2012), ImageSurfer[24], OsiriX[25] and IMOD[26].

As with niche image-analysis software, even generalist open-source platforms are developed by researchers involved in particular biology projects, and therefore, although they are usable for most tasks, they emphasize aspects of image analysis that are most relevant for the work they had been designed to do. For example, Fiji is currently the tool of choice in analysis of electron-microscopy data, Icy offers unique features for behavioral analysis, cell segmentation and cell tracking, and Vaa3D is heavily biased toward neurobiology applications and, together with BioimageXD, offers the best facilities for 3D visualization (see below). The extensive online resources available for most platforms are an excellent place to explore the strengths of the software. Similarly, the active mailing lists and chat-room channels run by the projects can be used to engage in direct interaction with the software's dedicated developers. Many innovative solutions are published and extensively cited, which offers traditional means of evaluating the impact of the tools.

ImageJ (originally called NIH Image) occupies a unique position in the landscape of open-source tools because it has been in use for the longest period of time and, notably, has always been free. It is the most popular and widespread multipurpose image-analysis tool[27–29]. Researchers have written hundreds of plug-ins and macros within this software to accomplish various image-processing and image-analysis tasks in different application areas. One of the main reasons for the success of this tool is that scientists can leverage ImageJ's infrastructure and dissemination while focusing on developing just the application-specific algorithm at hand. This extensibility has made it a favorite among both developers and end users, and the general architecture was later adopted by platforms such as Icy and Vaa3D. Owing to its rich history and pioneering status, ImageJ can perform a wide variety of common (and many specialized) image-processing and image-analysis tasks, particularly in the life sciences, and the user community has grown very large. ImageJ is constantly evolving to meet the needs of the scientific community, as evidenced by the community-driven ImageJ2 project (http://developer.imagej.net), which is developing the next generation of ImageJ to include support for many features such as large multidimensional image support, a more flexible data model and improved developer resources.

One challenge in extensible, interoperable, community-driven software projects is the proliferation of features, options, plug-ins and macros. In some contexts, having too many options is just as difficult as having too few because selecting among them can be overwhelming. A major challenge for the scientist is not only in picking which tool to use, but where to begin within a tool that offers many solutions. To address this challenge, the Fiji ImageJ distribution was developed to offer ImageJ bundled together with plug-ins and features specifically tailored to the microscopy community and to offer new functionality for microscopy analysis[20].

The plug-ins are distributed through an integrated updater system that facilitates rapid feedback between plug-in users and authors. Additionally, Fiji bundles several scripting languages that can be used in combination with algorithm libraries written in Java such as ImgLib (see below), to rapidly prototype new algorithms and facilitate productive interdisciplinary collaboration between the biology and computer science research communities.

CellProfiler is a flexible multipurpose open-source image-analysis tool for the life sciences that has a track record of utility and success[22]. It contains highly curated modules that can be mixed and matched to create customized image-analysis pipelines for a variety of biological systems including cells, colonies and *Caernorhabditis elegans*. Designed to accommodate high-throughput analysis, it is used to address several application areas, including intensity and morphology measurements, phenotype scoring by machine learning and object tracking.

More recently, comprehensive image-analysis tools focusing on 3D or high-dimensional image data have emerged. BioImageXD[18] and Icy[19], both based on the Visualization Toolkit (VTK) and the Insight Toolkit (ITK), offer many options for 2D and 3D analysis and are extensible via plug-ins and macros. BioImageXD can be used to generate immersive visualizations by recording flight paths through 3D renderings of multidimensional image data sets[18]. Icy is the youngest project in the open-source bioimage-analysis field and as such aims to combine the very best features of the existing tools as documented by the integrated pipeline building tools, interactions with microscopy hardware and a seamless update system. Vaa3D provides state-of-art algorithms for registration, visualization and analysis of large-scale multidimensional imagery of complex biological systems (embryos and brains) and mapping to anatomical atlases for further integrative analysis[21].

Table 1 | Summary of open-source software discussed in this Review

Software name	Primary function	Website
µManager	Image acquisition	http://www.micro-manager.org/
ScanImage	Image acquisition	http://www.scanimage.org/
OMERO	Image database	http://www.openmicroscopy.org/
Bisque	Image database	http://www.bioimage.ucsb.edu/bisque/
OMERO.searcher	Image content search	http://murphylab.web.cmu.edu/software/searcher/
Bio-Formats	Image format conversion	http://www.openmicroscopy.org/
ImageJ	Image analysis	http://rsbweb.nih.gov/ij/
Fiji	Image analysis	http://www.fiji.sc/
BioImageXD	Image analysis	http://www.bioimagexd.net/
Icy	Image analysis	http://icy.bioimageanalysis.org/
CellProfiler	Image analysis	http://www.cellprofiler.org/
Vaa3D	Visualization and image analysis	http://www.vaa3d.org/
FarSight	Visualization	http://www.farsight-toolkit.org/
VTK	Bioimaging library	http://www.vtk.org/
ITK	Bioimaging library	http://www.itk.org/
OpenCV	Bioimaging library	http://opencv.willowgarage.com/wiki/
WND-CHARM	Machine learning	http://code.google.com/p/wnd-charm/
PSLID	Machine learning	http://pslid.org/
Ilastik	Machine learning	http://www.ilastik.org/
CellProfiler Analyst	Machine learning and data analysis	http://www.cellprofiler.org/
PatternUnmixer	Machine learning	http://murphylab.cbi.cmu.edu/software/PatternUnmixer2.0/
CellOrganizer	Machine learning, modeling and visualization	http://cellorganizer.org/
KNIME	Workflow system	http://www.knime.org/

Extensibility, interoperability and code sharing. Image-analysis software is ideally extensible and interoperable, as no one tool can offer every function a researcher needs. For example, in the case of time-lapse cell analysis, a researcher may need to run many analysis routines including image denoising and deconvolution, cell identification and tracking, and measurement using advanced machine-learning analysis. The biological community has benefited from many recently constructed interfaces between image-analysis software packages. It is now possible, for example, to use ImageJ for image processing and CellProfiler for cell tracking because of a link between them, enabling a more automated workflow[30]. Fiji is closely collaborating with the ImageJ2 project to provide a next-generation ImageJ with improved low- and high-level functionality. The benefits of this coupling are numerous, particularly in the areas of performance, multidimensional support and modularity. The current data model of ImageJ is largely limited to two or three dimensions, making multidimensional analysis difficult. The *n*-dimensional (*n*D) model of ImageJ2 and Fiji will better support multidimensional image analysis including new modalities, such as fluorescence lifetime microscopy in ImageJ2 or selective plane illumination microscopy, uniquely available in Fiji[31]. Similarly, improvements in the ImageJ2 core will allow for code that is more centralized, easier to repackage and share with other packages in Fiji and other platforms using Java. It is encouraging to note that all the open-source platforms discussed in this section are explicitly developing ways to share data and code as only interoperability will enable biologists to mix and match the best features of each platform to solve the daunting image-analysis challenges.

Choosing an image-analysis platform. All these options for image analysis and image informatics in general (**Table 1** and many more not listed) bring up a key challenge for the user: how to choose which tool to use. Both among commercial and open-source software and across these categories there is a daunting array of options with substantial feature overlap. With some aspects of image informatics workflows other than image analysis, this decision is much easier and can be based on feature sets alone as often only one tool will have the features a user would want for data acquisition or storage. However in image analysis choosing between tools often comes down to preference for familiar interface, ease of use and other intangible criteria, much in the same way a computer user chooses an operating system. The developer community fully realizes this challenge, which is why there is a big movement to develop software applications that make the choice easier or unnecessary. All the open-source

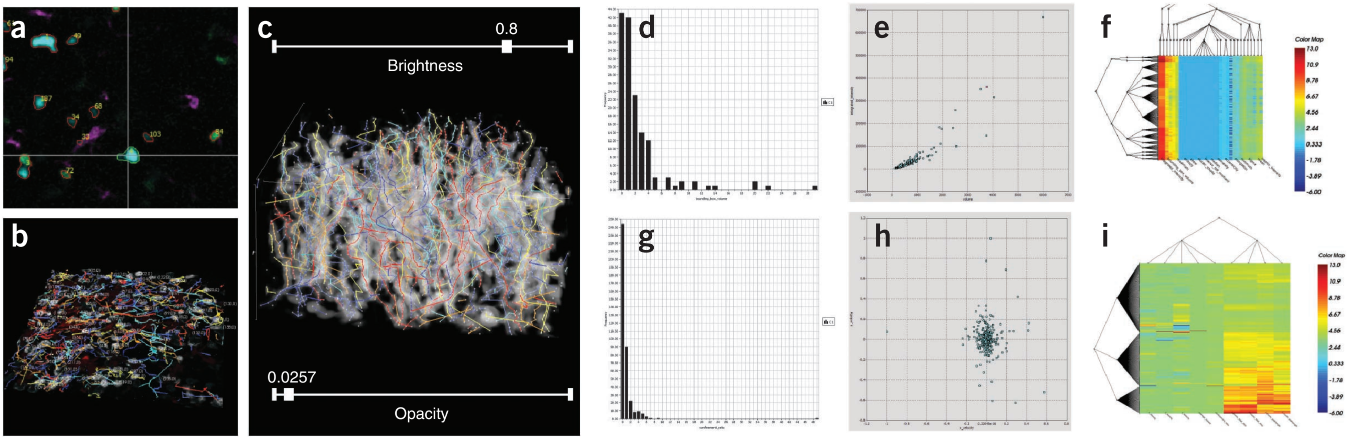


Figure 5 | Screenshots illustrating the image-analysis steps starting from a multichannel, multiphoton time-lapse movie culminating in a bioinformatics profiling of the extracted spatiotemporal data, using the FARSIGHT toolkit. (**a**) This movie (courtesy of E. Robey, University of California Berkeley) recorded 3D movements of thymocytes in an *ex vivo* preparation of a live developing mouse thymus at 2-min intervals, with wild-type thymocytes displayed in cyan, F5 thymocytes in green, and dendritic cells in violet. The first step is cell segmentation, shown as an orthogonal (*x, y, z* and time (*t*)) view. Cells are delineated and identified with numbers that correspond to rows of a table of cell measurements (data not shown). (**b**,**c**) The cell-tracking results are displayed in a 'beads on strings' view, showing the 3D movement paths of cells for detecting anomalies (**b**), and a '3D kymograph view', showing the same movement paths overlaid on a spatiotemporal (*x, y* and *t*) projection for convenience of assessing cell-tracking accuracy (**c**). (**d**) Histogram of cell-morphological measurements (size). (**e**) Scatter plots provide a visual cytometric summary of pairs of measurements. (**f**) Coifman bi-cluster plots organize the cell data into groups based on the cytometric data. (**g**) Histogram of cell tracking measurements (track tortuosity). (**h**) Scatter plot view of pairs of cell-track measurements. (**i**) Coifman bi-cluster plot organizing the cell tracks into groups based on the track-based measurements. Bi-cluster modules are courtesy of R. Coifman (Yale University) and L. Carin (Duke University).

software applications described above use code from other applications in the form of libraries (see below) and in some cases one platform can even be run inside another. For example all of the programs discussed use Bio-Formats to open (and in some cases save) their data, and many, such as Icy and CellProfiler, directly call ImageJ plug-ins. It is, however, not possible to run Icy inside ImageJ. It is important that the projects develop their software architecture in a way that makes integration mutual as we discuss below. Moving beyond a typical monolithic approach means the choice for a user is easier if they do not have to decide between two alternate software packages; they can use both. Workflow systems (discussed below) that are just beginning to emerge in the biological image analysis community offer even more flexibility. They enable calling each application as components in the analysis pipeline, allowing users to build their own virtual systems and select feature sets from many applications.

Image visualization

Although visualization of most 2D microscopy images is trivial using a variety of software packages, modern microscopy methods enable direct capture of *n*-dimensional data (several data channels across three spatial dimensions and time). The spatial dimensions alone can be very large as the addition of a computer-controlled stage allows the researcher to perform step-and-repeat microscopy, imaging large regions of tissue (millimeters to centimeters) as a montage with sub-micrometer resolution[32,33]. Techniques such as serial section microscopy enable this montaging to extend to the axial dimension[34]. In selective plane illumination microscopy, the dimensionality expansion becomes multiplied as multichannel, multiview data sets are recorded over time for observing dynamics of protein expression and localization in a live developing embryo. Below we review some of the visualization approaches currently used in microscopy. We also direct the reader to the prior *Nature Methods* supplement on visualizing biological data, which included a Review specifically devoted to the visualization of image data[35].

Multidimensional imaging techniques allow direct comparison of labeled biological entities in full spatial and temporal context, avoiding the need to piece together separate observations. However, the benefits come at the cost of more complex data storage, visualization and analysis needs. With modern software tools and contemporary desktop graphics hardware, a three-dimensional multichannel image cube can be projected onto the screen at interactive speeds, allowing the user to examine the data from any chosen angle and zoom factor. The speed usually results from the exploitation of hardware graphics processing engines and careful software optimization such as that used in the Vaa3D software[21]. When the entire image series can be loaded in computer memory, Vaa3D can be used to produce real-time 5D rendering. An alternate strategy used, for example, by ImageJ's View5D uses cross-sectional views that display only the data currently viewed at the appropriate scale. This strategy can also be easily realized on the web, allowing Google Maps–style browsing through massive multidimensional image volumes[36]. Interactive 3D visualization of large data sets remains a challenge. One obvious approach is hierarchical visualization, a method that combines both global and local 3D rendering windows in memory and an additional navigation window for extremely large image files (for example, > 8–10 gigabytes per image stack to terabytes of image data sets).

Another source of increased dimensionality is systematic imaging of large numbers of reporters in independent samples, for example, dozens of antibody probes or thousands of 3D–registered image stacks organized as different channels. For such cases, software systems[21] now allow users to use a spreadsheet-based color manager to efficiently blend and map data channels into the RGB space of computer monitors for 3D rendering.

A whole new set of visualization needs emerge after automated image analysis, for example, segmentation (**Fig. 5a**), tracking (**Fig. 5b**), feature extraction (**Fig. 5c**) and modeling. Segmentation and tracking operations produce massive amounts of multivariate data, commonly termed 'features'. The subsequent analysis, visualization and interpretation of the extracted image features from multidimensional data requires specially adapted tools, including scatter plots, histograms, dendrograms, bi-clustering panels, database query forms and progression displays. The FARSIGHT toolkit actively links these displays with each other and with the multidimensional image data, enabling exploration of relationships among objects and among their derived numerical data. It also enables editing of segmentation and tracking results and quantitative analysis techniques, such as the automatic identification of outliers or groups among the features. Vaa3D can act as a platform for further analysis of the feature spaces. For instance, a simultaneous segmentation and recognition approach built upon Vaa3D[37], which deforms the atlas of *C. elegans*[38] to best fit to a newly acquired 3D image, allows more robust identification of single cell identities at a lower rate of segmentation error, compared to the widely used 3D watershed-type segmentation. Vaa3D handles various image-analysis tasks using a plug-in interface, with which many modules such as image registration and segmentation, image input and output, and others can be glued together easily.

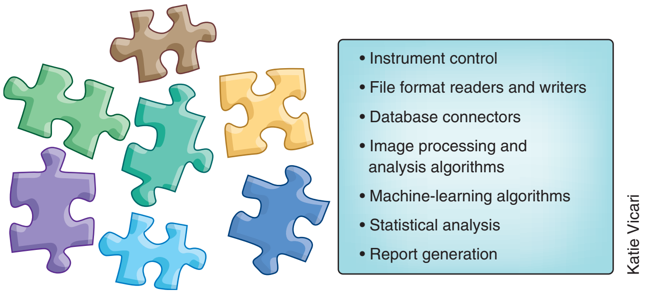
Multidimensional imaging is in a rapid phase of development, both from the perspective of the imaging hardware technology and the visualization and analysis software. The challenges stemming from growth in data volume to terabytes and beyond has motivated major extensions to image-processing toolkits discussed below.

BIOIMAGING LIBRARIES AND TOOLKITS

The image informatics community needs not only robust image-processing software but also imaging libraries, or toolkits (**Fig. 6**). A library is a collection of low-level algorithms that can be used, by those with programming experience, either directly or as pieces upon which end-user software applications can be built. These toolkits serve a critical role by allowing new approaches to be rapidly and flexibly tested before incorporation into end-user software. They tend to be modular and enable addition of functionality to end-user applications.

Although some commercial image-oriented libraries exist, such as the Image Processing toolbox in Matlab, the majority of bioimaging libraries are free and open-source. Open-source code is particularly helpful for libraries because of their potential to iterate and evolve rapidly with input from the scientific community.

A variety of image-analysis libraries exist, differing in their programming language compatibility, memory requirements, types of images supported, algorithmic focus, speed and level of computer science background required (**Table 2**). When evaluating in practice whether a particular library or toolkit provides the functionalities needed for a given image-processing workflow, it is convenient to start by visiting the introductory tutorials that most of these software projects host. They typically showcase the common uses of the library and provide an overview of the library's capabilities. Online forums and mailing lists are another valuable resource. They tend to be quite active and are an effective mechanism for getting feedback on the capabilities of the software, practical advice on how to apply it to specific problems and in some cases even ideas or assistance in modifying the source code to support new functionality. In addition to those described in more detail below, some examples of actively used libraries include Vision with Generic Algorithms (VIGRA; http://hci.iwr.uni-heidelberg.de/vigra/), a computer vision library with an emphasis on customizable algorithms and data structures, and EBImage[39], a modular package that leverages the R environment (http://www.r-project.org/) to segment cells and extract quantitative cellular descriptors.


• Instrument control
• File format readers and writers
• Database connectors
• Image processing and analysis algorithms
• Machine-learning algorithms
• Statistical analysis
• Report generation

Katie Vicari

Figure 6 | Bioimaging libraries and toolkits are available to cover a range of functionalities.

Among open-source bioimaging libraries, VTK and ITK have a prominent role. Both VTK and ITK are designed as a collection of data-processing units, filters that take input data and produce processed output data. These filters can be combined into processing pipelines that provide the flexibility and adaptability required by unexpected processing needs. Both ITK and VTK are written in C++ and then are wrapped into other languages, in particular Python, Tcl and Java. These two toolkits provide support for managing data sets that are too large to fit in the main computer memory by partitioning the input data into smaller segments that are then processed one by one. VTK's main focus is on visualizing 2D and 3D images and geometrical meshes with a large variety of rendering techniques, together with 3D widgets that facilitate user interactions with the objects being visualized. It also provides a collection of methods for visualizing information, such as charts, plots, trees and clusters. ITK is a complementary library focused on actual data processing rather than visualization; it is common to find ITK and VTK being used together by application developers. ITK provides one of the largest collections of image-analysis algorithms, in particular for image segmentation, image registration, image stitching and feature extraction. The toolkit supports *n*-dimensional images, with particular emphasis in two, three and four dimensions. ITK also provides support for a large variety of image file formats, including JPEG 2000, HDF5 and TIFF images larger than 4 gigabytes. Among the many bioimaging applications that are based on ITK or VTK are Icy, BioImageXD, Go-Figure (http://gofigure2.org/), Vaa3D and FARSIGHT. When dealing with very large data sets that demand the use of distributed parallel computation platforms, such as clusters and supercomputers, it is common as well to use ParaView (http://paraview.org/), an open-source application built on top of VTK, which provides a client-server architecture.

OpenCV (http://opencv.willowgarage.com/wiki/) is an open-source library that provides a rich set of image-analysis algorithms in the domain of computer vision, for native languages (C++, C and Python). OpenCV offers, for example, feature-extraction algorithms that can identify notable structures from images,

Table 2 | Summary of image-analysis libraries

Library	Language	Compatible with	Image dimensions	Computer science level for users	Algorithmic focus
VTK	C++	Tcl, Python and Java	2D and 3D	Medium	Filtering and visualization
ITK	C++	Python and Java	*n*D	Advanced	Segmentation and registration
OpenCV	C++	Python and Java	2D plus time	Medium	Feature extraction, tracking and visualization
ImgLib	Java	Java	*n*D	Advanced	Segmentation and registration
VIGRA	C++	Python	*n*D	Medium	Filtering
EBImage	R	R	2D and 3D	Basic	Analysis and segmentation

feature matching and tracking algorithms that can follow moving objects in video sequences and calibration algorithms for correlating objects from 3D space with features that they project into the 2D plane of an imaging sensor. It has been used especially in the automated monitoring of phenotype behavior of animal models, which often requires analysis of hundreds of hours of video.

Because many of the widely used generalist platforms for bioimage analysis use or are written in Java, a Java library called ImgLib was developed, primarily under the Fiji project. ImgLib[20] enables the software developer to concentrate on the essence of the image-analysis algorithm, transforming a mathematical formulation into a single piece of code that will run on images of any dimensionality (1D, 2D, 3D and *n*D), type (8-bit, 12-bit, 16-bit or complex types) or storage strategy (memory, disc or internet server). Several major Java-based software projects (Fiji, ImageJ2, Konstanz Information Miner (KNIME), OMERO and Icy) are in the process of migrating toward using ImgLib as the primary or at least alternate means of image data representation, which will in the future substantially improve their interoperability.

Indeed, using a common library for representing image data enables easily moving algorithmic solutions between platforms or running different parts of the processing pipeline using different software on the same data. The issue of interoperability is crucial for long-term progress in bioimage informatics, and general software libraries are one mechanism to achieve maximum integration among diverse platforms geared toward particular use cases (for example, software designed for automated processing of hundreds of thousands of images in high-throughput screens, such as CellProfiler, versus software that emphasizes processing very large images from multidimensional microscopy, such as Fiji). In addition, many bioimaging libraries have recently been linked by bridges, for example, between OpenCV and ITK, and between VTK and ITK. This empowers application developers to build upon the functionalities provided by both libraries.

Another way to achieve interoperability even among platforms that rely on fundamentally incompatible programming languages (Java versus C) is to agree on common formats to store the image data and the results of computational analysis so that one software output can be seamlessly used as input for another software package. The Bio-Formats library enables usage of diverse software packages by enabling each one of them to import any proprietary format and to rely on the standardized OME-TIFF format for data exchange. As bioimage data become increasingly complex, there is a need for additional development of agreed-upon data structures capable of efficiently capturing the analysis results and presenting them for downstream analysis. Integrative platforms for image-data management such as OMERO or BISQUE or workflow tools such as KNIME will be indispensable for creating diverse ecosystems of cooperating bioimage-analysis tools.

MACHINE LEARNING

Machine learning has been powerfully applied to experiments involving microscopy images[40,41] and is typically defined as a field concerned with creating programs whose performance improves with experience. Although non-experts may be intimidated by the concepts of machine learning, in reality these tools often require less expertise than tools that use manually configured algorithms (**Fig. 7**).

Machine learning in bioimaging is mainly used for classification—classification of either individual pixels, regions of interest in images (for example, cells) or whole images. Whereas model-based image processing algorithms are often used to directly identify regions of interest (for example, nucleus, cytoplasm and background), machine learning can be an alternative approach to automatically classify pixels as belonging to each class, particularly in challenging cases. Ilastik (http://www.ilastik.org/) is one open-source tool that enables researchers to train a machine-learning algorithm to identify which pixels of an image belong to which class of interest, based on the researcher providing example regions of each. At the image level (whether a whole field of view or a portion thereof showing a biological entity, such as a cell), biologists often need to decide to which class a particular image belongs, such as whether a protein is in one organelle or another, or whether a cell has undergone differentiation or transformation.

To address many such problems, an experimenter provides two or more collections of images and specifies quantities (called features) that are thought or expected to be relevant to the problem (such as the number, size or shape of cell nuclei). Image-processing methods are then used to calculate the features, and machine-learning methods are used to decide the values of the features that distinguish between the classes. However, often the quantities to measure are unclear a priori, are difficult for an experimenter to define sufficiently to get robust measurements or do not in practice achieve the desired goal. In these cases, an alternative is to extract a large set of features and let the computational methods determine, or 'learn', an optimal set to use. In either case, the output is a trained classifier that can be used to sort new images into the predefined classes, often better than a human can, as well as a set of statistics about the classification accuracy the system achieved[42–44].

The features calculated as the basis for applying machine-learning algorithms to images can be derived by first identifying structures of biological interest (for example, nuclei and cell boundaries), but they can also report on properties of the image as a whole such as textures, pixel statistics and factors in polynomial equations that approximate the image. The variety of image content these features represent directly determines the types of image changes to which the machine-learning program will be sensitive.

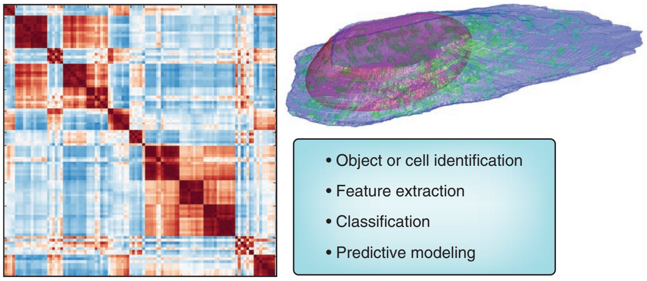


Figure 7 | Machine learning. Application areas in which machine learning is used in bioimaging.

Three general categories of tasks can be carried out using the features: statistical comparisons, supervised learning and unsupervised learning. Statistical comparisons operate on the feature distributions directly, such as to determine whether two sets of images are statistically distinguishable or which image is most representative of a set, for example, to choose an image for publication, as can be done using the typical image chooser functionality in PSLID.

In supervised learning, the biologist defines relationships between images, such as by grouping together example images for different classes (a classification problem) or specifying the concentration of a drug that each sample received (a regression problem). In both cases, the program determines automatically which of the features are informative for distinguishing the classes or estimating the extent of response. Although many machine-learning programs for microscopy rely on some image preprocessing to first identify and isolate (that is, segment) regions of interest such as cells or nuclei, classification has also been achieved without segmentation[45,46]. Thus, machine learning can be applied to biological images with little or no input other than the initial sorting of images into classes. When instead the desired output is a continuous variable, regression methods, which have many concepts in common with classification, are used. The training images are grouped by their known outputs to construct a regression function (a 'standard curve'). Examples include dose-response curves (responses to a drug at increasing concentrations)[47] and time points in a time series[44].

When using supervised machine learning, it is important to ensure that the trained system can generalize to new images. Different subsets of the training images are used so that the system does not become too customized to the training images, a phenomenon known as overtraining. Problems are sometimes encountered if the training set does not adequately represent the variability present in the experimental samples (such as if all of the training images are acquired on one day and there is variation from day to day). Elimination of selection bias and systematic bias in the training set is in the hands of the experimenter and is crucial in machine learning because of the absence of an a priori model.

Sometimes, the classes into which images should be grouped are unknown (or uncertain). In this case, cluster analysis, a form of unsupervised machine learning, can be used to find groups within data. For example, clustering has been used to group drugs by their effects[48] and proteins by their subcellular patterns[49].

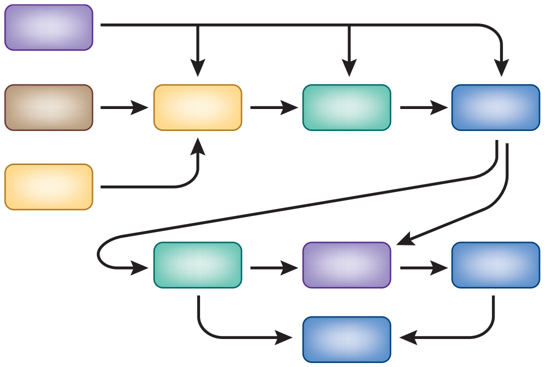
Whether to use supervised or unsupervised machine learning for a particular bioimaging problem is often a difficult choice[41]. It hinges mainly on the extent to which the user believes that all important classes (patterns) are already known but also on the confidence with which the user believes that the training images assigned to each class are representative of that class for all other images that will be analyzed. If labeled images are available (or can be obtained), a frequent approach is to first use supervised learning to determine how well the features can distinguish at least the major classes and then use unsupervised learning to attempt to discover new classes, subclasses within known classes or variation within a class over time.

Supervised and unsupervised methods can be combined, an approach referred to as semi-supervised learning. The idea is to use some information about classes and then extend it using the data. For example, images might be grouped based on the identity of a treatment (supervised), and these samples can then be clustered into morphologically defined classes (unsupervised). An example of this used weighted neighbor distance using compound hierarchy of algorithms representing morphology (WND-CHARM) and time points throughout the lifespan to train a classifier to assign individual *C. elegans* a physiological age score[44].

There are also several approaches in which input can be introduced during the learning process. For example, in CellProfiler Analyst[50], the biologist is presented with machine classifications of new cells based on their previous input and is given the opportunity to correct errors. This new information is used to retrain the classifier. If there is a high degree of uncertainty in the classifications, a machine learner can ask the user to label selected examples in a process of active learning[41]. This process can potentially be made fully automatic, as demonstrated in ref. 51, where a classifier was used to control an acquisition system for optimally acquiring additional examples to increase its accuracy.

Machine learning has been proven useful for several purposes in bioimaging, but there are some limitations. Classifiers typically do not transfer well between cell types or between different imaging systems; retraining must often be performed. A related problem is that classification and clustering systems can only 'represent' the patterns they are trained on. An alternative is to try to represent a subcellular pattern or cell morphology using image-derived generative models. Given multiple images of a given pattern, a model can be trained to capture the essence and the variation in that pattern and can synthesize new images that in a statistical sense are drawn from the same distribution as the training images. This approach has only recently been applied in bioimaging; however, reasonably accurate methods for building models for nuclear and cell shape and some organelles are available.

A final limitation of machine-learning approaches is that they are not typically used to handle mixtures of classes. Take, for example, the application of machine learning to identify to which organelle a particular protein localizes after machine training on example images showing localization in each distinct compartment. A protein can be anywhere on a continuum between fully in one compartment to fully in one or more other compartments. This leads classifiers to produce arbitrary results when presented with images of mixed patterns and can lead clustering algorithms to form 'chained' clusters (or to artificially divide the continuum into pieces). A solution to this problem is to directly estimate how much of a given protein is in each of multiple 'fundamental' compartments (for example, 10% is in lysosomes and



- Visual modeling of entire image-processing and analysis workflow
- Documentation of the workflow, enabling reproducible research
- Linking disparate software tools
- Automatic translation between required formats
- Workflows developed by experts serve as templates for novice users
- Integration with other information resources

Figure 8 | Workflow systems. Benefits of using a workflow system.

90% is in the Golgi complex). This can be done given training images of proteins that are known to be in just one compartment (such as the 'marker' proteins used in training classifiers), representing each image by the amount of fluorescence in distinct object types and using various standard unmixing methods. This 'supervised unmixing' approach has been successful[52] and can be done using the open-source PatternUnmixer software. It is even possible to do 'unsupervised unmixing' by estimating both the fundamental patterns and the mixing fractions[53].

WORKFLOW SYSTEMS

Workflow systems for data processing and analysis have begun to serve an important role across many fields in biology, including bioimaging. Workflow-based tools allow researchers to flexibly and intuitively model data processing and analysis protocols and integrate a diverse array of tools without writing complex scripts or being constrained to application-focused, monolithic tools (**Fig. 8**). As such, these tools often address a complementary niche, where researchers are not repeating the same analysis day after day but instead are exploring and testing solutions or need to adapt to a broad array of applications. These workflow tools have become increasingly attractive because the need to process and analyze data in a sophisticated way is spreading from specialists to the majority of modern biologists. Being able to reproducibly and intuitively read, transform, process and analyze data has become a necessary skill, very much like word processing 20 years ago.

Workflow systems can enable a field to transition from requiring that experts manually string together many single-purpose software tools, to enabling non-experts to create a seamless workflow using a single integrated tool. Workflow tools have begun to serve the bioimaging community well: both expert users who know how to tweak every parameter of an algorithm, to the broader user base needing an intuitive user interface. 'Visual programming' is the most common approach to user-friendliness in this domain, enabling access to a variety of functionality (for example, image processing and data mining) while also giving access to multiple data sources (for example, chemical, biological and textual). Other advantages are the ability to readily document and disseminate an analysis, supporting reproducible research and rapid sharing of new approaches.

Several workflow systems have emerged over the past decade or so. Several companies offer workflow tools, such as SAS' Enterprise Miner and IBM/SPSS Clementine, offering tools for more general data mining and statistical analysis. More focused on the life science market is Accelrys' Pipeline Pilot, which offers a combination of tools that address cheminformatics and image analysis, and also have some data analysis capabilities. These tools are proprietary but have been offered at a steep discount for academics and are thus often used for teaching and academic research. Still, the rapid spread and continual evolution of bioimaging-related software has made it challenging for a single commercial entity to offer solutions that cover the spectrum of needs in this domain and integrate all of the relevant technology. Given the availability of state-of-the-art open-source tools for individual steps of the bioimaging workflow, it is therefore no surprise that in recent years open-source workflow systems have gained popularity. The workflow system itself has taken a backstage role as a data- and tool-integration backbone and—much like is the case for Linux—companies are more willing to invest in open standards for such critical infrastructure pieces. Open-source workflow tools such as Taverna (http://www.taverna.org.uk/) and Galaxy (http://galaxy.psu.edu/) focus on bioinformatics, whereas KNIME is an option that serves an even broader set of domains, including business intelligence and predictive analytics. KNIME also has connections to other bioimaging tools, such as ImageJ, OMERO and CellProfiler, among others, allowing the creation of complex image-processing and analysis workflows. The use of workflow software in academic biological imaging is still very new, but these approaches are routinely used in pharmaceutical research and have great potential to be applied to large-scale, multistep analysis problems.

When considering what workflow system to use, or whether a workflow approach is even necessary, it is important to first define whether a monolithic approach or a framework is needed. Many of the tools discussed above allow for robust workflows to be created and run, such as a series of image-processing tasks in Icy or CellProfiler. The main difference between Icy and other similar applications and tools such as Taverna and KNIME is that the former are trying to do everything by themselves (sometimes incorporating other tools but not necessarily in a very flexible way). The latter are open frameworks that allow the easy integration of various other libraries and tools, in such a way that you can easily swap one tool for another one. Taverna does this by adopting a standard (web services), KNIME has its own open API and data-type format. Both are quite effective, but the advantage of the open API approach is that web services are a lot harder to 'archive' as web services can vary over time. In contrast, if you ran a workflow in KNIME 2.4.3 in 2008 you will still be able to run it in 2018 using that version of KNIME. When thinking about the steps involved in a workflow, there are two important steps: the steps in the image-analysis pipeline itself and the steps of using such image analysis in a sustainable way. For the steps in the pipeline it is important to emphasize the importance of any user being able to swap tools out easily and use a different tool or library for different tasks. The ability to visually document what one has done is also important. In thinking about the steps of using such image-analysis workflows in a sustainable way, the key issues addressed by a workflow system are reproducibility, archivability and the ability to share workflows.

It is important to note that a workflow system is not needed for many image-processing and analysis tasks, and many workflow needs can be addressed by the more monolithic approach in current image-analysis applications. One of the strengths of a platform such as KNIME is that it is a separate piece of technology from the software pieces that actually do the work. So KNIME concentrates on the modeling of the analysis 'pipeline' and allows the user to integrate whatever software libraries for the image loading, processing or analysis one wants to use (or other routines such as chemical modeling and text analysis). A user can even launch their own in-house toolbox for their preferred way to do an arbitrary image processing process.

The ability to access cutting-edge technology as different modules within a workflow system also yields a challenge: version control. The ability to reproduce an analysis precisely is critical in most scientific domains. Proprietary tools have the ability, at least in theory, to ensure that workflows continue to produce the same results with progressive updates to the software, but this is not always the case. Further, those without licenses for the software cannot reproduce another researcher's analysis, and the closed-source nature of the software limits the ability of others to rely on the software being available in perpetuity. For open-source tools, version control and reproducibility are at least feasible. This is commonly addressed by taking snapshots of the particular version of the code bases on which an end-user application depends and storing those code bases in a combined repository or by simply referring to a specific tagged version in the official repository of the code bases. For example, the end-user application Slicer 4.0 is built by pointing to a specific version of ITK, VTK, Python and many other tools. Vaa3D and Icy are also built by pointing to specific versions of ITK. The multiplatform configuration tool CMake makes this process straightforward by providing the functionality of 'superbuilds' and 'external projects'. Tools that heavily rely on external toolkits (in the extreme case, via WebService calls) allow a workflow itself to be archived, although the tools it calls may cease to function or may produce different results over time. Tools such as KNIME offer users a choice in this respect by offering to call webservices or alternately to freeze a certain state, ensuring workflow reproducibility in the future.

CONCLUSIONS

As technology progresses, modern biologists must become increasingly familiar with computational techniques and software tools. Researchers who use microscopy are no exception to this rule; fortunately bioimaging software has recently rapidly developed in terms of both functionality and usability. Emphasis on functionality stems from the fact that developers of bioimaging software are typically embedded in, or have strong ties to, experimental biology labs. Thus, the software produced by this developer community is usually in direct answer to current biology needs and often in response to new emerging problems outside the scope of commercial interest. Usability has often lagged behind, but there is increasing recognition of its value to biomedical research[54].

Although the initial focus of each software project in this domain was solely to address a single step in the bioimaging workflow, the bioimaging software community has recently begun to address an important aspect of usability: interoperability. The open-source bioimaging software community has begun to communicate and collaborate, assisted and reflected by conferences such as BioImage Informatics and the Cold Spring Harbor Laboratory's Automated Imaging and High-Throughput Phenotyping meeting. As the community gains momentum, connections among independent software projects have begun to be prioritized, and some have already been completed, as highlighted here. These connections greatly ease researchers' work by reducing the need to tediously transfer data between multiple software packages.

As described in Commentaries in this issue[54,55], there is a great need to not only collaborate on bioimaging ideas and approaches but also on software coding itself, including consensus on best practices and standards, software quality control, documentation, training, maintainability and sharing of modular code. The many activities where the developers and users interact and develop their skills—for example, tutorials, conferences and hackathons—promote the progress of the software by fostering rapid innovation and interdisciplinary thinking. In fact, the community of researchers surrounding most open-source bioimaging software projects is usually much more important than the software itself. Software without community is a static resource of limited lifespan, whereas an active community adapts continuously to new problems. These highly networked collaborations are a common property of open-source software projects, multiplying the value of a simple software resource and enabling the attack of scientific problems too large or interdisciplinary to be addressed in individual labs[56]. There are many challenges facing the imaging community, such as tracking and annotating large multidimensional data sets and retaining and sharing complex heterogeneous data sets, but also opportunities for pooling resources, such as in software coding or in testing and validation using crowd sourcing. All of these challenges necessitate the development of a robust bioimaging informatics platform.

The last 20 years have seen extraordinary biological advances driven by new biological imaging tools, many of which directly relied on computational methods. The future of biological imaging innovation depends even more squarely on developing image informatics solutions. Continued advances in bioimaging computational approaches will serve not only as the foundation for new imaging methods but as the catalyst for new biological discovery that would not otherwise be possible. Resources invested in the development and maintenance of important bioimaging software applications as well as connections among them promise to yield great dividends to the thousands of biologists relying on bioimaging.

ACKNOWLEDGMENTS

We acknowledge our respective funding sources and members of our laboratories for feedback and useful comments, in particular A. Merouane and A. Narayanswamy of the Roysam lab for their assistance in preparing figures, and L. Kamentsky and M. Bray of the Carpenter lab for useful input and edits on the manuscript. A.E.C. and K.W.E. were supported by US National Institutes of Health grants R01 GM089652 (to A.E.C.) and RC2 GM092519 (to K.W.E.).

DISCLAIMER

Certain trade names, company products and open-source software products are identified in this review. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that such products are necessarily fit for their intended purpose.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

Published online at http://www.nature.com/doifinder/10.1038/nmeth.2084. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Peng, H. Bioimage informatics: a new area of engineering biology. *Bioinformatics* **24**, 1827–1836 (2008).
2. Gustafsson, M.G. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. *Proc. Natl. Acad. Sci. USA* **102**, 13081–13086 (2005).
3. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science* **319**, 810–813 (2008).
4. Hess, S.T., Girirajan, T.P. & Mason, M.D. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* **91**, 4258–4272 (2006).
5. Jones, S.A., Shim, S.H., He, J. & Zhuang, X. Fast, three-dimensional super-resolution imaging of live cells. *Nat. Methods* **8**, 499–508 (2011).
6. Planchon, T.A. *et al.* Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination. *Nat. Methods* **8**, 417–423 (2011).
7. Edelstein, A., Amodaj, N., Hoover, K., Vale, R. & Stuurman, N. Computer control of microscopes using µManager. *Curr. Protoc. Mol. Biol.* **92**, 14.20.11–14.20.17 (2010).
8. Lin, H.P., Vincenz, C., Eliceiri, K.W., Kerppola, T.K. & Ogle, B.M. Bimolecular fluorescence complementation analysis of eukaryotic fusion products. *Biol. Cell* **102**, 525–537 (2010).
9. Pologruto, T.A., Sabatini, B.L. & Svoboda, K. ScanImage: flexible software for operating laser scanning microscopes. *Biomed. Eng. Online* **2**, 13 (2003).
10. Conrad, C. *et al.* Micropilot: automation of fluorescence microscopy-based imaging for systems biology. *Nat. Methods* **8**, 246–249 (2011).
11. Allan, C. *et al.* OMERO: flexible, model-driven data management for experimental biology. *Nat. Methods* **9**, 245–253 (2012).
12. Kvilekval, K., Fedorov, D., Obara, B., Singh, A. & Manjunath, B.S. Bisque: a platform for bioimage analysis and management. *Bioinformatics* **26**, 544–552 (2010).
13. Wu, L., Faloutsos, C., Sycara, K.P. & Payne, T.R. Feedback adaptive loop for content-based retrieval. in *Proceedings of the 26th International Conference on Very Large Data Bases* (Morgan Kaufmann Publishers Inc., 2000).
14. Goff, S.A. *et al.* The iPlant Collaborative: cyberinfrastructure for plant biology. *Frontiers in Plant Science* **2**, 34 (2011).
15. Glory, E. & Murphy, R.F. Automated subcellular location determination and high-throughput microscopy. *Dev. Cell* **12**, 7–16 (2007).
16. Ljosa, V. & Carpenter, A.E. Introduction to the quantitative analysis of two-dimensional fluorescence microscopy images for cell-based screening. *PLoS Comput. Biol.* **5**, e1000603 (2009).
17. Lakowicz, J.R. *Principals of Fluorescence Spectroscopy*. (Academic Press, New York, 1999).
18. Kankaanpää, P. *et al.* BioImageXD: an open, general-purpose and high-throughput image-processing platform. *Nat. Methods* **9**, 683–689 (2012).
19. de Chaumont, F. *et al.* Icy: an open bioimage informatics platform for extended reproducible research. *Nat. Methods* **9**, 690–696 (2012).
20. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
21. Peng, H., Ruan, Z., Long, F., Simpson, J.H. & Myers, E.W. V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. *Nat. Biotechnol.* **28**, 348–353 (2010).
22. Carpenter, A.E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7**, R100 (2006).
23. Fiala, J.C. Reconstruct: a free editor for serial section microscopy. *J. Microsc.* **218**, 52–61 (2005).
24. Feng, D. *et al.* Stepping into the third dimension. *J. Neurosci.* **27**, 12757–12760 (2007).
25. Rosset, A., Spadola, L., Ratib, O. & Osiri, X. An open-source software for navigating in multidimensional DICOM images. *J. Digit. Imaging* **17**, 205–216 (2004).
26. Kremer, J.R., Mastronarde, D.N. & McIntosh, J.R. Computer visualization of three-dimensional image data using IMOD. *J. Struct. Biol.* **116**, 71–76 (1996).
27. Collins, T.J. ImageJ for microscopy. *Biotechniques* **43**, 25–30 (2007).
28. Abramoff, M., Magalhaes, P. & Ram, S. Image processing with ImageJ. *Biophotonics International* **11**, 36–42 (2004).
29. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
30. Kamentsky, L. *et al.* Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* **27**, 1179–1180 (2011).
31. Preibisch, S., Saalfeld, S., Schindelin, J. & Tomancak, P. Software for bead-based registration of selective plane illumination microscopy data. *Nat. Methods* **7**, 418–419 (2010).
32. Tsai, C.L. *et al.* Robust, globally consistent and fully automatic multi-image registration and montage synthesis for 3-D multi-channel images. *J. Microsc.* **243**, 154–171 (2011).
33. Preibisch, S., Saalfeld, S. & Tomančák, P. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics* **25**, 1463–1465 (2009).
34. Saalfeld, S., Fetter, R., Cardona, R. & Tomancak, P. Elastic volume reconstruction from series of ultrathin microscopy sections. *Nat. Methods* **9**, 717–720 (2012).
35. Walter, T. *et al.* Visualization of image data from cells to organisms. *Nat. Methods* **7**, S26–S41 (2010).
36. Saalfeld, S., Cardona, A., Hartenstein, V. & Tomančák, P. CATMAID: collaborative annotation toolkit for massive amounts of image data. *Bioinformatics* **25**, 1984–1986 (2009).
37. Qu, L. *et al.* Simultaneous recognition and segmentation of cells: application in *C. elegans*. *Bioinformatics* **27**, 2895–2902 (2011).
38. Long, F., Peng, H., Liu, X., Kim, S.K. & Myers, E. A 3D digital atlas of *C. elegans* and its application to single-cell analyses. *Nat. Methods* **6**, 667–672 (2009).
39. Pau, G., Fuchs, F., Sklyar, O., Boutros, M. & Huber, W. EBImage–an R package for image processing with applications to cellular phenotypes. *Bioinformatics* **26**, 979–981 (2010).
40. Shamir, L., Delaney, J.D., Orlov, N., Eckley, D.M. & Goldberg, I.G. Pattern recognition software and techniques for biological image analysis. *PLoS Comput. Biol.* **6**, e1000974 (2010).
41. Murphy, R.F. An active role for machine learning in drug development. *Nat. Chem. Biol.* **7**, 327–330 (2011).
42. Murphy, R.F., Velliste, M. & Porreca, G. Robust numerical features for description and classification of subcellular location patterns in fluorescence microscope images. *J. VLSI Signal Process.* **35**, 311–321 (2003).
43. Nattkemper, T.W., Twellmann, T., Ritter, H. & Schubert, W. Human vs machine: evaluation of fluorescence micrographs. *Comput. Biol. Med.* **33**, 31–43 (2003).
44. Johnston, J., Iser, W.B., Chow, D.K., Goldberg, I.G. & Wolkow, C.A. Quantitative image analysis reveals distinct structural transitions during aging in *Caenorhabditis elegans* tissues. *PLoS ONE* **3**, e2821 (2008).
45. Huang, K. & Murphy, R.F. From quantitative microscopy to automated image understanding. *J. Biomed. Opt.* **9**, 893–912 (2004).
46. Shamir, L. *et al.* Wndchrm – an open source utility for biological image analysis. *Source Code Biol. Med.* **3**, 13 (2008).
47. Loo, L.H., Wu, L.F. & Altschuler, S.J. Image-based multivariate profiling of drug responses from single cells. *Nat. Methods* **4**, 445–453 (2007).
48. Perlman, Z.E. *et al.* Multidimensional drug profiling by automated microscopy. *Science* **306**, 1194–1198 (2004).
49. Chen, X. & Murphy, R.F. Objective clustering of proteins based on subcellular location patterns. *J. Biomed. Biotechnol.* **2005**, 87–95 (2005).
50. Jones, T.R. *et al.* Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc. Natl. Acad. Sci. USA* **106**, 1826–1831 (2009).
51. Jackson, C., Glory-Afshar, E., Murphy, R.F. & Kovacevic, J. Model building and intelligent acquisition with application to protein subcellular location classification. *Bioinformatics* **27**, 1854–1859 (2011).
52. Peng, T. *et al.* Determining the distribution of probes between different subcellular locations through automated unmixing of subcellular patterns. *Proc. Natl. Acad. Sci. USA* **107**, 2944–2949 (2010).
53. Coelho, L.P., Peng, T. & Murphy, R.F. Quantifying the distribution of probes between subcellular locations using unsupervised pattern unmixing. *Bioinformatics* **26**, i7–i12 (2010).
54. Carpenter, A.E., Kamentsky, L. & Eliceiri, K.W. A call for bioimaging software usability. *Nat. Methods* **9**, 666–670 (2012).
55. Cardona, A. & Tomancak, P. Current challenges in open-source bioimage informatics. *Nat. Methods* **9**, 661–665 (2012).
56. Nielsen, M. Reinventing Discovery: The New Era of Networked Science. (Princeton University Press, 2011).
57. Linkert, M. *et al.* Metadata matters: access to image data in the real world. *J. Cell Biol.* **189**, 777–782 (2010).
58. Larson, S.D. & Martone, M.E. Ontologies for neuroscience: what are they and what are they good for? *Front. Neurosci.* **3**, 60–67 (2009).
59. Plant, A.L., Elliott, J.T. & Bhat, T.N. New concepts for building vocabulary for cell image ontologies. *BMC Bioinformatics* **12**, 487 (2011).
60. Swedlow, J.R. Finding an image in a haystack: the case for public image repositories. *Nat. Cell Biol.* **13**, 183 (2011).

Corrigendum: Biological imaging software tools

Kevin W Eliceiri, Michael R Berthold, Ilya G Goldberg, Luis Ibáñez, B S Manjunath, Maryann E Martone, Robert F Murphy, Hanchuan Peng, Anne L Plant, Badrinath Roysam, Nico Stuurmann, Jason R Swedlow, Pavel Tomancak & Anne E Carpenter

***Nat. Methods* 9, 697–710 (2012); published online 28 June 2012; corrected after print 20 July 2012.**

In the version of this article initially published, Nico Stuurman's last name was incorrect. The error has been corrected in the HTML and PDF versions of the article.

Corrigendum: Biological imaging software tools

Kevin W Eliceiri, Michael R Berthold, Ilya G Goldberg, Luis Ibáñez, B S Manjunath, Maryann E Martone, Robert F Murphy, Hanchuan Peng, Anne L Plant, Badrinath Roysam, Nico Stuurman, Jason R Swedlow, Pavel Tomancak & Anne E Carpenter

***Nat. Methods* 9, 697–710 (2012); published online 28 June 2012; corrected after print 20 July 2012; corrected after print 29 August 2012.**

In the version of this article initially published, the disclaimer was omitted. The error has been corrected in the HTML and PDF versions of the article.