

# BioImageXD: an open, general-purpose and high-throughput image-processing platform

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**BioImageXD puts open-source computer science tools for three-dimensional visualization and analysis into the hands of all researchers, through a user-friendly graphical interface tuned to the needs of biologists. BioImageXD has no restrictive licenses or undisclosed algorithms and enables publication of precise, reproducible and modifiable workflows. It allows simple construction of processing pipelines and should enable biologists to perform challenging analyses of complex processes. We demonstrate its performance in a study of integrin clustering in response to selected inhibitors.**

Bioimaging has emerged as one of the key tools in biomedical research. New imaging devices are appearing rapidly, producing large multidimensional images with several data channels in medium-to-high throughput. Specialized software is required to extract interpretable results from such data. Despite recent and ongoing progress in both commercial and academic software, availability of user-friendly tools often remains a limiting factor, especially in complex and high-throughput applications<sup>1–3</sup>. We formed a multidisciplinary consortium to develop bioimage processing software (**Supplementary Methods**) that is (i) open: has open-source code; (ii) extensive: has many features; (iii) usable: is inexpensive, easy to use and fast; (iv) adjustable: all features are adjustable and combinable into pipelines; (v) applicable: especially for validated batch processing of multidimensional time-lapse data; and (vi) extendable: with a modular design that allows addition of new functionality.

We now report the release of version 1.0 of BioImageXD (<http://www.bioimagexd.net/>). We explain for each of our design criteria why we consider them important and describe how BioImageXD fulfills them. We then compare BioImageXD to other software. Finally we demonstrate the capabilities of

BioImageXD in practice with user-friendly, automated, multiparametric image analyses of the movement, clustering and internalization of integrins.

## Criteria in BioImageXD development

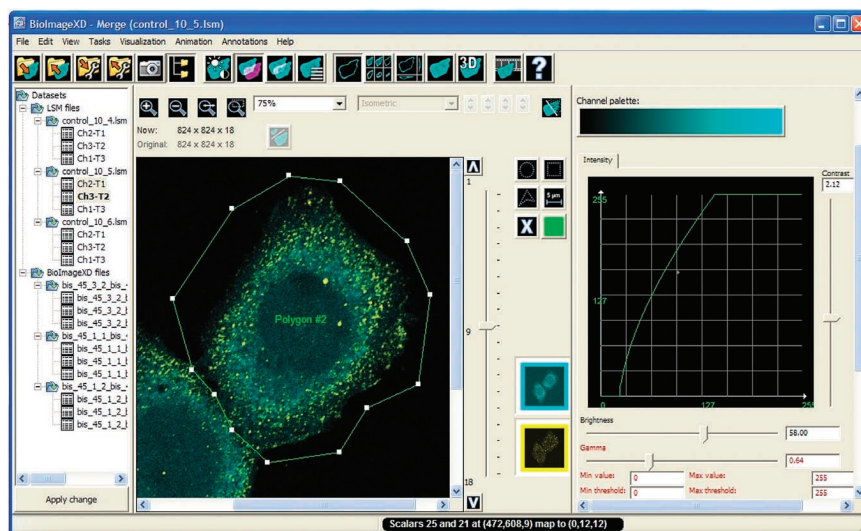
**Open.** Our goal was to develop software that is transparent, with open and documented source code and no undisclosed algorithms or hidden processing the user is unaware of or cannot control. It is our view that software for scientific data analysis, in contrast to engineering, is best when it is not a 'black box'. Different implementations of the same method can often give different results, the reasons for which only become clear by comparing the source codes. Increasingly (and correctly in our view), disclosure of source code for image-processing methods and algorithms is recommended in scientific research<sup>4–6</sup>. Publication of scientific software tools also often requires the source code (the method) to be open, as is the norm for all other protocols in sample preparation, experimental procedure and data analysis. BioImageXD (**Fig. 1**) is released under a GNU General Public License version 2 (<http://www.gnu.org/licenses/gpl-2.0.html>); the source code is fully open.

Subsampling of image data in software may occur without the user's knowledge, for instance in the interest of user-interface responsiveness, and may affect data interpretations. Although BioImageXD can also subsample image data, subsampling is never done automatically and is always clearly indicated. All processed image data are written into new files by user action, with processing information automatically saved, making workflows retraceable and minimizing inadvertent processing. A concise user guide is provided with the software.

**Extensive.** We sought to develop software with a comprehensive collection of features for processing,

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**Figure 1** | BioImageXD GUI. Screenshot shows toolbars and menus (top), a file tree of the loaded images (left), and the currently active visualization (middle), showing here a two-channel merged image with a user-defined region of interest. On the right are settings for the currently active task, showing here the graphical intensity transfer function, a central feature in accurate image adjustment.



visualization and analysis. Bioimaging applications today are greatly varied, and a single software package should be able to meet as many needs as possible, without requiring extra programming. BioImageXD has been designed to be a general-purpose processing program, featuring approximately 220 tools (**Supplementary Table 1**). Processing tools include deconvolution, registration, 32 tools for basic mathematical and logical processing, and six noise-reduction algorithms. Visualization tools include an interactive three-dimensional (3D) mode with 11 simultaneously visible modules for creating volume renderings, surface renderings, 3D measurements and so on (**Fig. 2**, **Supplementary Fig. 1** and **Supplementary Video 1**). Movies can be created with an animator supporting two different combinable techniques: keyframes and virtual camera flight paths (**Supplementary Fig. 2** and **Supplementary Videos 2** and **3**). Analysis tools feature both voxel-based and object-based approaches. For the latter, 17 methods are available for segmenting image data into quantifiable objects (**Supplementary Fig. 3** and **Supplementary Video 4**) based on thresholding, watershed, region growing and active contours. A 3D motion tracking algorithm can follow the movement of different features, such as a few cells, or hundreds of segmented objects in a cell (**Supplementary Video 5**).

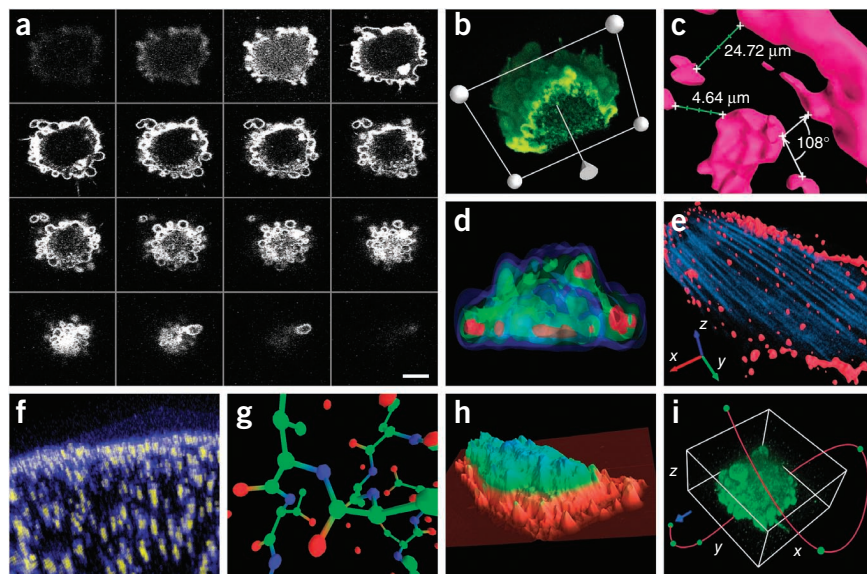
Many scientific journals have guidelines for setting up the intensity-transfer function (used to adjust, for instance, brightness and contrast)<sup>5</sup>. The function is represented graphically in

BioImageXD (**Fig. 1**), enabling the user to check this graph and evaluate possible data loss (which happens in principle every time brightness or contrast, for example, is increased).

Studies of colocalization (whether two markers occupy the same spatiotemporal location) are very common but are often poorly executed in part owing to tool inaccessibility<sup>7,8</sup>. In BioImageXD, colocalization can be visualized with maps and histograms, and quantified with 54 parameters. Three statistical significance methods<sup>9–11</sup>, automatic threshold determination<sup>9</sup>, and both object-based and pixel-based colocalization are supported.

**Usable.** Our aim was to make BioImageXD inexpensive and simple to install and run on any common computer. One of the bottlenecks of bioimage processing is that the required software often comes with an expensive and restrictive license policy, limiting availability to isolated workstations that may also control microscopes. BioImageXD can be used free of charge on any computer with either 32-bit or 64-bit Windows (XP, Vista or 7), Mac OSX (10.6 or 10.7) or modern Linux operating systems. The software consists of a single package that is simple to install. Most features work

without specific system requirements, but if processor-intensive tasks progress slowly, BioImageXD can subsample images on loading



**Figure 2** | Examples of visualizations created with BioImageXD. (a) Gallery mode for viewing 3D images as 2D slices. Scale bar, 10  $\mu\text{m}$ . (b) Volume rendering of 2D slices into a 3D image, shown here with a cutting plane. (c) Surface rendering, shown here with 3D distance and angle measurements. (d) Several semitransparent surfaces created at the same time. (e) Volume rendering (blue actin filaments) and surface rendering (red integrins) displayed together, shown here with a 3D axis marker. (f) Volume rendered cell-surface proteins. (g) A 3D visualization of molecular models; detail of an integrin I-domain model<sup>37</sup>. (h) Warp scalar rendering of a cell showing atomic force microscopy data in three dimensions. (i) The 3D renderings can be animated in various ways, such as by specifying a 'flight path' (red line) for a virtual camera.

(this is clearly indicated and easily undone). Data sets larger than the computer's RAM can be visualized and processed.

Bioimaging software should be easy and efficient to use, with an intuitive graphical user interface (GUI)<sup>12</sup>. The GUI of BioImageXD is based on a single large window (**Fig. 1**) with an unchanged layout. Advanced settings are always available but are colored red to warn novice users (**Fig. 1**). Additional assistance is offered in simple on-screen instructions (examples are shown in **Supplementary Figs. 3 and 4**). For added performance, computationally demanding parts of the code have been implemented as platform-native optimized libraries, written in C++. However, the software architecture and implementation still leave room for improvement in performance.

**Adjustable.** To enable transparent and reproducible science, our goal was to develop software in which parameters for all methods and algorithms are individually adjustable in the GUI, identified by their correct technical name, but not at the expense of making the GUI too cluttered. In BioImageXD, the layout and on-screen color-coding and instructions (which include accepted inputs and outputs) also aid in pipeline assembly, which extends the flexibility of software for more complex image-processing needs. Such procedure lists can be created in BioImageXD without programming skills, assembling many methods and algorithms into tunable, human-readable pipelines.

**Applicable.** Wide-field and confocal fluorescence microscopy are among the most commonly used methods in bioimaging. Combinations of multiple channels, three dimensions, time series and images acquired in high throughput are becoming the norm, as systems biology imaging approaches gather momentum<sup>13–16</sup>. We therefore wished to develop software that is applicable to these situations and also to general 2D image processing. In BioImageXD, we integrated three-dimensional, multichannel and time-lapse support into the implementations of all processing tools as well as the possibility to automate batch processing of large amounts of data. Images are not loaded into memory upon opening them. Instead, image data are read only on demand, for efficient memory use when switching between data sets or handling large time series. Channels are processed separately to avoid confusion. Any command pipeline created in BioImageXD can be used for batch processing. The batch processor has a GUI (**Supplementary Fig. 4**), can run several pipelines simultaneously and aggregates results into spreadsheets, thus facilitating high-throughput data processing. BioImageXD is already used for multiwell applications in some laboratories, but pipelines cannot exchange data, and the retention of spatial information on multiwell plates is not yet supported. We are working on these aspects. High-throughput applications are a major development area for BioImageXD, with ongoing projects aiming at performing complex analyses interactively in real time with high-throughput image-acquisition platforms.

Validations tools are an important aspect of bioimaging software applicability. For complex and high-throughput applications, in particular, it is often difficult to verify results manually. BioImageXD can create simulated images, with a tool that can in its current iteration simulate particles with various distributions in relation to presegmented cell shapes and various levels of noise. The simulated data can be used to validate segmentation, motion tracking and other procedures in many applications.

**Extendable.** As scientific research continuously spawns new questions, requiring implementation of new or modified tools to answer them, bioimaging software should build upon and engineer compatibility with existing libraries and tools<sup>17</sup>. We therefore designed BioImageXD to have modular, extendable software architecture, primarily written in Python for simplicity and extendibility, and to leverage the open-source libraries Visualization Toolkit (VTK)<sup>18</sup> and Insight Segmentation and Registration Toolkit (ITK)<sup>19</sup>. It has a large adjustable feature set, with input from common microscopy and generic image file formats, including the Open Microscopy Environment (OME) TIFF format<sup>20</sup>, and output in open standard formats (VTK XML image, TIFF, OME-TIFF and comma-separated value spreadsheets). Ideally, the architecture of BioImageXD should also facilitate widespread use of new algorithms.

### Comparing BioImageXD to other software

We feel it is useful to compare the main characteristics of BioImageXD to those of other biological image-processing software. However, we note that such comparisons are difficult to make completely fairly and accurately: the tools may have fundamentally different goals, and in these comparisons we could only cover a subset of all the available software packages. Despite these limitations, we defined a testing protocol based on our six design criteria and compared BioImageXD to other open-source software, BioImage Suite<sup>21</sup>, ImageJ (Fiji, <http://fiji.sc/>), Vaa3D (ref. 22) and VisBio<sup>23</sup>, and also to proprietary software, Imaris (Bitplane), Fluoview (Olympus), Volocity (PerkinElmer), ZEN Lite (Zeiss) and Matlab plus Image Processing Toolbox (MathWorks). For these comparisons, we listed qualitative descriptions of features and characteristics of the software for each of our six criteria, assessed the number of features on a four-point scale and measured the time it took to perform certain basic operations (**Table 1** and **Supplementary Tables 2–4**).

Closed-source software products have a different design philosophy than that of BioImageXD. By definition, they cannot fulfill our first criterion of openness; furthermore, their usability, adjustability and especially extendibility are often restricted. BioImageXD compared favorably to the proprietary software we considered in the number of features and performance speed, but the proprietary software may offer more stability and maturity.

Other open-source software projects are as open and extendable as BioImageXD but are often less extensive or applicable. However, comparisons are difficult, as different projects have different aims. For instance, ImageJ is a generic platform for processing individual images, with an easy plugin architecture, resulting in many tools developed by a large community, whereas BioImageXD is a single package, combining the algorithmic and 3D visualization power of ITK and VTK with a user-friendly GUI.

Matlab, in contrast, has a different philosophy from all the other software, as its use requires programming. We included it in the comparisons to make them more representative of the different approaches available.

The open-source programs we considered may be better suited than BioImageXD for some specific applications, such as filament tracing, image annotation, manual manipulation of individual images or processing of bright-field images. However, BioImageXD often compares favorably to them in speed and has more well-integrated features, especially in areas such as 3D visualization, segmentation and tracking. BioImageXD also offers



**Table 1** | Summary for comparison of BioImageXD to other software

	Open	Extensive	Usable	Adjustable	Applicable	Extendable
BioImageXD (1.0RC3)	••••	••••	••••	••••	••••	••••
BioImage Suite (3.01)	••••	••	•••	•••	•	••••
ImageJ (Fiji) (Madison, Wisconsin, USA, updated 02/2012)	••••	••	••	•••	••	••••
Vaa3D (previously V3D) (2.707)	•••	••	•••	•••	•	••••
VisBio (3.40RC1)	••••	•	••	••	•	•••
Matlab (7.9.0.529) plus IPT (6.4) (MathWorks)	••	••	•••	•••	•	••
Imaris (7.4.0) (Bitplane)	•	•••	•••	••	••	••
FluoView (3.01.01.09) (Olympus)	••	••	••	••	•	•
Volocity (6.0.1) (PerkinElmer)	•	••	•	•••	••	•
ZEN Lite (2011) (Zeiss)	••	•	••	•	••	•

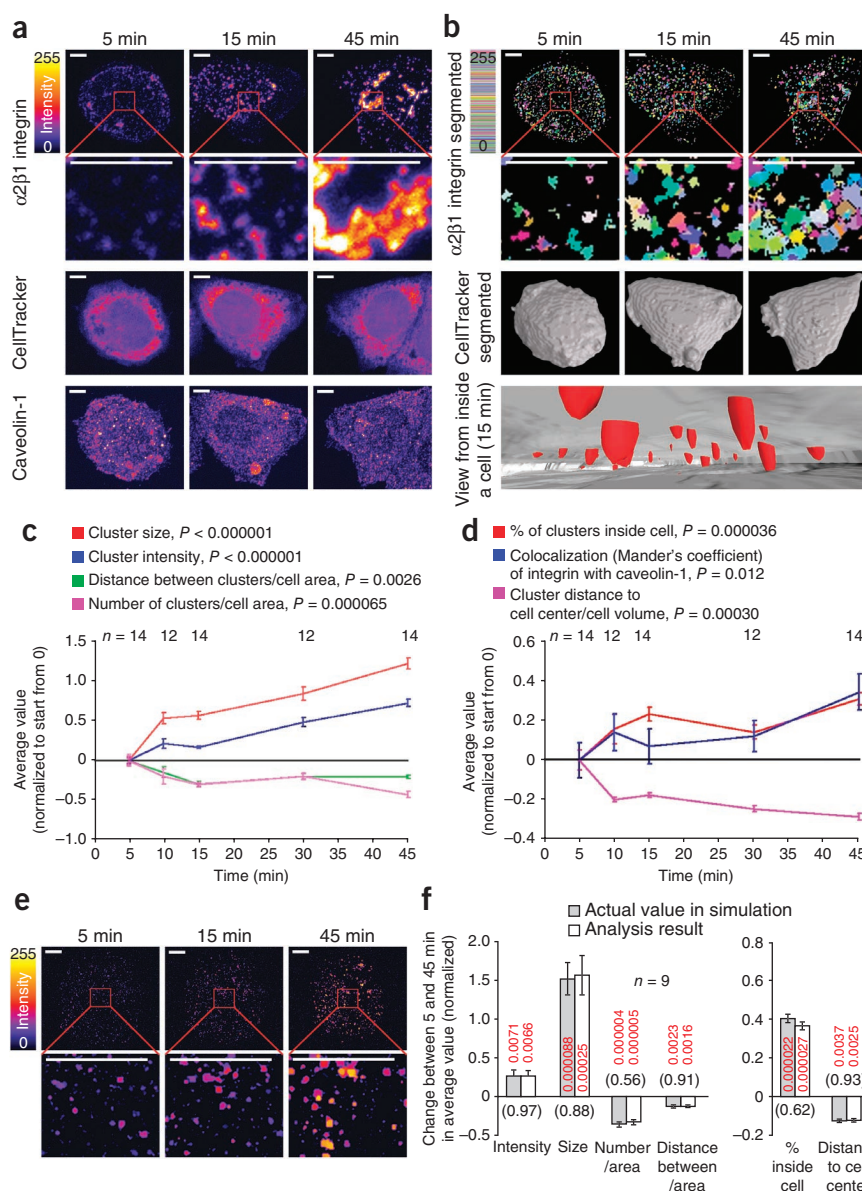
For each of our six software design criteria, each software was given a score from 1 to 4 (indicated by dots), based on how many subcategories it fulfilled for that criterion. These comparisons have limitations: they were carried out for a limited set of software and were done from the point of view of the design criteria used for BioImageXD. Other software may have fundamentally different design criteria. IPT, Image Processing Toolbox.

simulation tools and user-friendly support for high-throughput command pipelines, unlike many other open-source platforms. We also compared the performance of BioImageXD to that of other software for a specific practical example of image analysis.

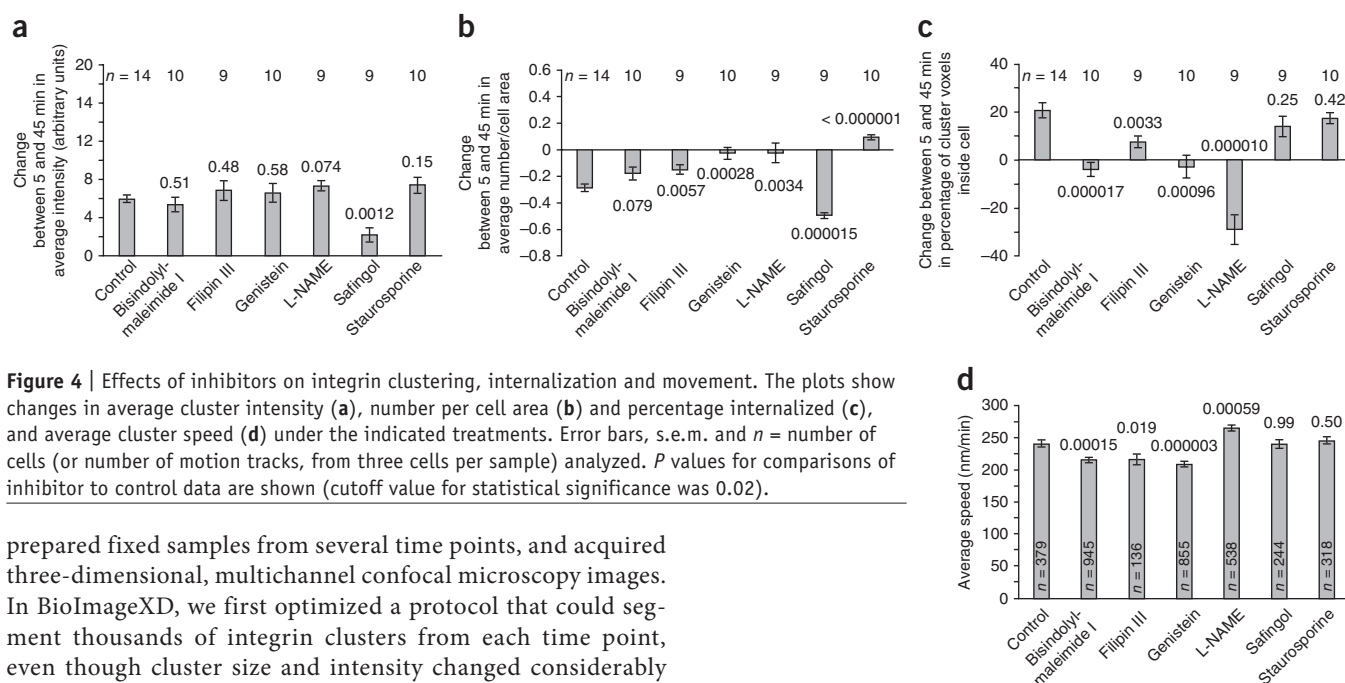
### Practical example: analyzing cell-surface receptors

As a proof of concept for BioImageXD, we undertook a challenging quantitative imaging study of integrin clustering, movement and internalization. Integrins anchor cells to their surroundings, and their intracellular domains are connected to the cytoskeleton, signaling networks and the endocytosis machinery<sup>24</sup>. Integrin  $\alpha 2\beta 1$ , a human collagen receptor, forms clusters after binding to multivalent ligands, such as collagen fibrils<sup>25</sup>. Lateral movement of  $\alpha 2\beta 1$  on the cell surface may also contribute to collagen fibril organization<sup>26</sup>. Furthermore,  $\alpha 2\beta 1$  is often internalized upon binding to soluble multivalent ligands or antibodies<sup>27</sup>. Internalization of  $\alpha 2\beta 1$  seems to take place with a unique mechanism compared to other integrins, but the entry pathway is incompletely known<sup>28</sup>.

We induced clustering of  $\alpha 2\beta 1$  integrin with fluorescently conjugated antibodies,



**Figure 3** | Analyzing cell-surface receptor clustering and internalization. **(a)** Maximum intensity projections of confocal microscopy images of sample cells. Each data channel is shown separately, as indicated. **(b)** Maximum intensity projections of segmented integrin clusters (top two rows, color bar shows arbitrary colors assigned to segmented objects), 3D surface renderings of the cell membrane defined from CellTracker staining, smoothed with Gaussian and median filtering (middle), and 3D surface rendering from inside the cell, illustrating internalization of integrin clusters (red; bottom). **(c)** Average integrin cluster size, intensity, number and distance to each other. **(d)** Integrin internalization, colocalization with caveolin-1 and distance to cell center. In **c** and **d** all parameters were normalized to begin with 0, and  $P$  values are shown for changes between 5 min and 45 min. **(e)** Sample images of simulated integrin clustering used to validate the analyses. **(f)** Using simulated data, changes between 5 min and 45 min of the indicated parameters.  $P$  values in red are for change over time, and  $P$  values in parentheses indicate statistical significance between actual values and corresponding analysis results. Error bars, s.e.m. ( $n$  values indicate number of cells or simulations analyzed). Scale bars, 5  $\mu\text{m}$ .



**Figure 4** | Effects of inhibitors on integrin clustering, internalization and movement. The plots show changes in average cluster intensity (**a**), number per cell area (**b**) and percentage internalized (**c**), and average cluster speed (**d**) under the indicated treatments. Error bars, s.e.m. and *n* = number of cells (or number of motion tracks, from three cells per sample) analyzed. *P* values for comparisons of inhibitor to control data are shown (cutoff value for statistical significance was 0.02).

prepared fixed samples from several time points, and acquired three-dimensional, multichannel confocal microscopy images. In BioImageXD, we first optimized a protocol that could segment thousands of integrin clusters from each time point, even though cluster size and intensity changed considerably with time, and the clusters often touched each other. We then calculated four central parameters: size, intensity, number and distance between clusters. The latter two depended on cell size (Supplementary Fig. 5), so we developed an additional protocol for using a CellTracker fluorescent stain to calculate cell surface area. As expected, cluster size and intensity increased over time, whereas cluster number and distance between them decreased (Fig. 3a–c).

To analyze internalization of the clusters, we developed a protocol that, simply put, used the previously segmented cell membrane and calculated how many of the integrin clusters were inside it. Internalization increased over time (Fig. 3d). We confirmed the result in several ways. First, we quantified cluster distance to the center of the cell, normalized for cell volume. As internalization increased, this distance decreased. Second, we compared the results to differential staining before and after cell permeabilization, and examined the effect of different membrane definition thresholds, all supporting our analysis (Supplementary Fig. 6). Finally, as the internalization of  $\alpha 2\beta 1$  integrin has been suggested to involve caveolin-1 (ref. 27), we included a third fluorescent marker for caveolin-1, and quantified the colocalization between it and  $\alpha 2\beta 1$ . As expected, the colocalization increased over time (Fig. 3d).

As there can be thousands of integrin clusters in a complex three-dimensional arrangement in every cell, checking analysis results manually, even for a single cell, is impossible. Therefore we created simulated images of integrin clustering and internalization with the simulation tools of BioImageXD and validated our analysis protocols with these data (Fig. 3e,f and Supplementary Fig. 7).

Using our validated analysis methods, we repeated all experiments with a selection of chemical inhibitors for various cellular signaling pathways (Fig. 4a–c and Supplementary Figs. 8–10).

Integrins move while clustering, and we analyzed this movement in living cells and tested the effects of the inhibitors. We applied tools for concatenating time series and registering them to compensate for focus drift and cell movement, and enhanced

the BioImageXD motion tracking algorithm to be able to track clustering objects (Fig. 4d and Supplementary Figs. 11 and 12). We confirmed the tracking functionality by repeating all analyses with a segmentation protocol producing much higher cluster numbers, with consistent results. Again, we created simulations to validate the analysis (Supplementary Fig. 13).

We carried out all analyses with the BioImageXD batch processor and aggregated the results into a single matrix (Supplementary Table 5), which revealed that the clustering, internalization and lateral movement of integrins are regulated by different molecular mechanisms. Sample data and instructions are available for reproducing the analyses, which are widely applicable in biomedical research (Supplementary Data 1 and 2).

To attempt the analyses with other software packages, we listed the critical steps in performing the analyses and checked whether it was possible to perform them with the other software. If yes, we calculated sample results. Obtaining accurate results was difficult owing to a lack or inflexibility of features (Supplementary Tables 6 and 7), and we thus conclude that BioImageXD can be used to conduct complex analyses that might otherwise be considered impractical. However, we note that this comparison was limited to the features included in the tested packages and did not cover all possible plugins or features that might be available to users. Also, expert users of different software might be able to obtain better results than we did.

## Discussion

The ethics of image processing is an important topic for the biological imaging community. With modern hardware and software, inaccurately manipulating image data is simple and tempting to the less scrupulous or poorly informed<sup>5,6,29,30</sup>. Openly described software implementations and full disclosure of the performed processing steps are probably the best ways to bring verifiability and transparency to the discipline<sup>4</sup>. Open-source projects have their challenges, such as maintaining continuity and rigorous organization, but these could be considered acceptable in order to achieve scientific reliability. Indeed, open-source programs

seem to be gaining popularity not only in science<sup>31,32</sup>, but also in general, as exemplified by the Linux operating system<sup>33</sup>. To fully participate in the open-source bioimaging community, a software must implement open standards for image data and numerical result formats, and be interoperable with existing tools. BioImageXD currently leverages VTK<sup>18</sup> and ITK<sup>19</sup>, and can read and write many image data formats, including the open standard OME-TIFF<sup>20</sup>. The BioImageXD consortium is open to collaborations, and we hope that in the future BioImageXD will be interoperable also with Java-based approaches and projects such as Icy<sup>34</sup>, CellProfiler<sup>35</sup>, ImageJ2 and Fiji<sup>36</sup>.

Other developers may define the criteria for a bioimaging platform differently than we do, but we believe our criteria are reasonable. BioImageXD is among the most versatile single software packages in areas such as 3D visualization and animation, colocalization analysis and segmentation. It also has fairly unique features, such as its operational logic, reading of image data only on demand, simulated data generation and fully integrated support for batch processing. BioImageXD can serve as core software where the need is greatest, in complex and high-throughput applications. It already has a strong user base, with 400 downloads of beta versions per month.

In our application of BioImageXD to the analysis of integrin clustering, we showed that BioImageXD can deliver in practice. The extensive feature set of BioImageXD, coupled to its GUI design, enabled the creation of complex protocols by new combinations of existing tools. Its extendable, open-source architecture enabled modifications that could, at best, be developed to the point of use within mere hours. The new analyses, combined with traditional colocalization analysis, could be carried out virtually simultaneously for hundreds of files with pipelines in the BioImageXD batch processor. We validated all new analyses with simulated image data, also created with BioImageXD. Simulated data are useful for more than just method validation. In complex cases, numerical results can be hard to understand in a biological context. If a model can be constructed that simulates the experimental data, different parameters become comprehensible because their effects can be explored. It is recommendable for researchers working with complex analyses to always validate their results with such simulation tools. BioImageXD should enable biologists who are not experts in computer science to efficiently carry out and verify a wide range of scientific image-processing tasks.

*Note: Supplementary information is available in the online version of the paper.*

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#### AUTHOR CONTRIBUTIONS

P.K. designed software and experiments, and wrote the manuscript; L.P. was head programmer; L.P. and J.P. programmed the software; P.K. and L.P. defined analysis protocols and did most analyses; S.T. and J.N. prepared samples, imaged and did some analyses; M.K. helped with software development; J.H. and V.M.

designed scientific applications and software strategies; and D.J.W. supervised software development.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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