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DEVELOPING BIOIMAGE INFORMATICS – FROM MICROSCOPY TO SOFTWARE SOLUTIONS – WITH α2β1 INTEGRIN AS A CASE STUDY

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Cover picture: Three-dimensional rendering of the center of a living cancer cell, as seen from inside the cell. Image acquired with a laser scanning confocal microscope and rendered with the BioImageXD software. Image acquisition and rendering by Pasi Kankaanpää.

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When I look upon my life, I do not think of impact factors, publications, science, work or money. I think of the people who care about me, and the people I care about - my family and my friends. This work is dedicated to all of you.

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Abstract

After the human genome was sequenced in 2003, the major task of biosciences became to find out the functions of different genes, and various bioimaging techniques became crucial research tools. Technological advances resulted in especially fluorescence-based light microscopy techniques to explode in popularity, but microscopy needed to transform from a qualitative science to a quantitative one. This transition resulted in a new field of science, bioimage informatics, which has been said to have the potential to revolutionize biosciences. This thesis describes an extensive, multidisciplinary body of work in bioimage informatics.

The first aim of the thesis was to develop protocols for four-dimensional confocal microscopy of living cells, one of the fastest growing methods of bioimaging. Human collagen receptor α2β1 integrin, an important molecule in several physiological and pathological processes, was used as a case study. Clear visualizations of the integrins moving, clustering and going inside cells were obtained, but there were no tools to quantify the image data. The second aim of the thesis then became to develop computer software capable of such quantification. At the same time, bioimage informatics was born, and specialized software was the most critical requirement of the new field. The main result of this thesis therefore became BioImageXD, a novel open source software package for visualizing, processing and analyzing multidimensional bioimages. BioImageXD grew to become one of the largest and most versatile in its field. It was published in a special bioimage informatics issue of Nature Methods, and became well known and widely used. The third aim of the thesis was to apply the developed methods to something more practical, and artificial silica nanoparticles were created, with antibodies against α2β1 integrin as "address labels". BioImageXD was successfully used to show that the nanoparticles have potential in targeted drug delivery applications.

A major underlying aim of this thesis was to promote the new and unknown science of bioimage informatics, and this aim was reached especially through BioImageXD and its numerous published applications. The work done in this thesis has considerable future potential, but bioimage informatics faces serious challenges. The field is too complex for the average biomedical researcher to master, and there is lack of recognition for the most important element, open source software development. Several improvements in these aspects are required, or alternatively researchers should consider obtaining bioimage informatics services from designated units or companies.

Pasi Kankaanpää. 2014. Biokuvainformatiikan kehittäminen – mikroskopiasta ohjelmistoratkaisuihin – sovellusesimerkkinä α2β1-integriini. Biokemian laitos ja MediCity-tutkimuslaboratorio, Turun yliopisto, Turku, ja Bioinformatiikan ja biorakenteiden kansallinen tohtoriohjelma (ISB), Turku. Annales Universitatis Turkuensis, Sarja AI, 491.

Tiivistelmä

Kun ihmisen genomi saatiin sekvensoitua vuonna 2003, biotieteiden päätehtäväksi tuli selvittää eri geenien tehtävät, ja erilaisista biokuvantamistekniikoista tuli keskeisiä tutkimusmenetelmiä. Teknologiset kehitysaskeleet johtivat erityisesti fluoresenssipohjaisten valomikroskopiatekniikoiden suosion räjähdysmäiseen kasvuun, mutta mikroskopian tuli muuntua kvalitatiivisesta tieteestä kvantitatiiviseksi. Tämä muutos synnytti uuden tieteenalan, biokuvainformatiikan, jonka on sanottu mahdollisesti mullistavan biotieteet. Tämä väitöskirja esittelee laajan, poikkitieteellisen työkokonaisuuden biokuvainformatiikan alalta.

Väitöskirjan ensimmäinen tavoite oli kehittää protokollia elävien solujen neliulotteiseen konfokaalimikroskopiaan, joka oli yksi nopeimmin kasvavista biokuvantamismenetelmistä. Ihmisen kollageenireseptori α2β1-integriini, joka on tärkeä molekyyli monissa fysiologisissa ja patologisissa prosesseissa, oli sovellusesimerkkinä. Työssä saavutettiin selkeitä visualisointeja integriinien liikkeistä, yhteenkeräytymisestä ja solun sisään siirtymisestä, mutta työkaluja kuvainformaation kvantitatiiviseen analysointiin ei ollut. Väitöskirjan toiseksi tavoitteeksi tulikin tällaiseen analysointiin soveltuvan tietokoneohjelmiston kehittäminen. Samaan aikaan syntyi biokuvainformatiikka, ja kipeimmin uudella alalla kaivattiin erikoistuneita tietokoneohjelmistoja. Tämän väitöskirjatyön tärkeimmäksi tulokseksi muodostui näin ollen BioImageXD, uudenlainen avoimen lähdekoodin ohjelmisto moniulotteisten biokuvien visualisointiin, prosessointiin ja analysointiin. BioImageXD kasvoi yhdeksi alansa suurimmista ja monipuolisimmista. Se julkaistiin Nature Methods -lehden biokuvainformatiikkaa käsittelevässä erikoisnumerossa, ja siitä tuli tunnettu ja laajalti käytetty. Väitöskirjan kolmas tavoite oli soveltaa kehitettyjä menetelmiä johonkin käytännönläheisempään. Tehtiin keinotekoisia piidioksidinanopartikkeleita, joissa oli "osoitelappuina" α2β1-integriinin tunnistavia vasta-aineita. BioImageXD:n avulla osoitettiin, että nanopartikkeleilla on potentiaalia lääkkeiden täsmäohjaussovelluksissa.

Tämän väitöskirjatyön yksi perimmäinen tavoite oli edistää uutta ja tuntematonta biokuvainformatiikan tieteenalaa, ja tämä tavoite saavutettiin erityisesti BioImageXD:n ja sen lukuisten julkaistujen sovellusten kautta. Väitöskirjatyöllä on merkittävää potentiaalia tulevaisuudessa, mutta biokuvainformatiikalla on vakavia haasteita. Ala on liian monimutkainen keskimääräisen biolääketieteen tutkijan hallittavaksi, ja alan keskeisin elementti, avoimen lähdekoodin ohjelmistokehitystyö, on aliarvostettu. Näihin seikkoihin tarvitaan useita parannuksia, tai vaihtoehtoisesti tutkijoiden tulisi harkita biokuvainformatiikan palveluiden hankintaa erillisistä yksiköistä tai yrityksistä.

Contents

Abbreviations

2D	two-dimensional
3D	three-dimensional
4D	four-dimensional
AFM	atomic force microscopy
CO_2	carbon dioxide
CT	computed tomography
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EM	electron microscopy
EV1	Echovirus 1
FLIM	fluorescence lifetime imaging
FM	fluorescence microscopy
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
IgA	immunoglobulin A
ITF	intensity transfer function
ITK	Insight segmentation and registration toolkit
LM	light microscopy
LSCM	laser scanning confocal microscopy
KNIME	Konstanz Information Miner
MAPK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
OME	Open Microscopy Environment
PALM	photo-activated localization microscopy
PBS	phosphate-buffered saline
PET	positron emission tomography
PSF	point spread function
RGB	red-green-blue
SAOS	osteogenic sarcoma
SIM	structured illumination microscopy
STED	stimulated emission depletion
STORM	stochastic optical reconstruction microscopy
TAT	Human immunodeficiency virus-1 transactivator protein
TIFF	Tagged image file format
TPA	12-O-tetradecanoylphorbol-13-acetate
TIRFM	total internal reflection fluorescence microscopy
VTK	Visualization toolkit
ZEN	Zeiss Efficient Navigation

Original Publications

This thesis is based on the following three original publications, hereafter referred to by the Roman numerals I-III. The publications are re-printed with permissions from the original publishers. In addition, 12 other peer-reviewed scientific publications were produced. These publications are not directly part of the thesis, but describe related studies, approaches and results. They are overviewed in the chapter "5. Related published work".

I. *Upla P, Marjomäki V, Kankaanpää P, Ivaska J, Hyypiä T, Van Der Goot FG, Heino J. 2004. Clustering induces a lateral redistribution of α2β1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol Biol Cell. 15: 625-636.*
This publication describes one of the first applications of four-dimensional (4D) laser scanning confocal microscopy of living cells in Northern Europe. Cell surface receptor molecules can be seen to cluster together, move along actin filaments, and go inside cells. The 4D imaging, image processing and video editing approaches used enabled these phenomena, describing a novel mechanism to activate a cellular entry process, to be clearly seen for the first time in living cells. The author of this thesis was responsible for carrying out most of the confocal imaging, and for writing the corresponding sections of the manuscript, as well as preparing most of the microscopic image and video material.

II. *Kankaanpää P, Paavolainen L, Tiitta S, Karjalainen M, Päivärinne J, Nieminen J, Marjomäki V, Heino J, White DJ. 2012. BioImageXD: an open, general-purpose and high-throughput image-processing platform. Nat Methods. 9: 683-689.*
During work for publication I it was discovered that specialized software needs to be developed for proper visualization and quantitative analysis of multidimensional bioimaging data. This publication describes the result of this development work: the BioImageXD software, one of the largest and most versatile software solutions available to tackle the major issue of lack of software in bioimage informatics. The author of this thesis is the corresponding author, and was responsible for designing the software, coordinating its development, designing the biological studies, analyses and software comparisons, writing the manuscript and preparing all images, videos and data. Practical software development was done in close collaboration with programming lead Lassi Paavolainen. As this publication describes an unusually large body of work, only a small part of it was covered by the original printed publication, and printable parts of the supplementary material have been reprinted in this thesis.

III. *Kankaanpää P, Tiitta S, Bergman L, Puranen A-B, von Haartman E, Lindén M, Heino J. 2014. Cellular recognition and macropinocytosis-like internalization of nanoparticles targeted to integrin α2β1. Submitted manuscript.*
This publication describes how the imaging methods and software tools developed in the previous publications were applied in the field of nanoscience, to develop

artificial nanoparticles capable of specifically entering cells via integrin cell surface receptors and potentially carrying therapeutic payloads. The author of this thesis was responsible for designing all the bioimaging experiments and analyses, carrying out most of the analyses, preparing all images and videos and writing most of the manuscript.

1. Background

1.1. Imaging in biomedical research

After the sequencing of the human genome was completed in 2003, biology entered a new era of informational biology. The genes of humans, and increasingly also the genes of many other species, were now known, and the major task at hand was to relate these genes to various cellular mechanisms and functions – to decipher their information content [1-3]. Approximately at the same time as the human genome project was nearing completion, another major development took place within biosciences: green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was shown to enable virtually any protein in any cell type to be made fluorescent and therefore specifically visible in microscopic images [4]. Now all the genes were known, and it was possible to make their protein products visible and see exactly what they were doing in a cell. Different bioimaging techniques quickly became very essential in the era of informational biology [3, 5-10].

Bioimaging can be thought to cover all kinds of techniques that produce images from biological and medical specimens. These techniques range from large scale methods of imaging entire organisms or organs, to high resolution imaging of single molecules [11]. For instance, in magnetic resonance imaging (MRI) images are formed from radio waves induced from hydrogen atoms by a strong magnetic field, in X-ray computed tomography (CT) X-rays from different angles are used to form three-dimensional images, and in positron emission tomography (PET) images are formed from positron-emitting radionuclides administered to the subjects. These techniques are used especially in medical imaging for clinical purposes, and they have a lower resolution. At the other end of the resolution spectrum are methods such as electron microscopy (EM), where an electron beam is used to form an image similarly to electromagnetic radiation in light microscopy, but the small de Broglie wavelength of the electrons enables much higher resolution than in light microscopy. Some EM techniques can produce also three-dimensional image data, but imaging biological samples under physiological conditions is not possible. Another high resolution method is atomic force microscopy (AFM), a mechanical imaging technique, where forces between a tiny tip and individual atoms in the sample are measured through the bending of a spring-like cantilever to which the tip is attached. Laser light reflected from the cantilever amplifies the bending movement so that it becomes large enough to measure. AFM can under optimal circumstances reach atomic lattice resolution, and unlike EM, it can be used under physiological conditions to study for instance the surface topography of a living cell. While both the medical and the high resolution imaging techniques are highly important, methods based on light microscopy (LM) are probably the most significant bioimaging techniques in the era of informational biology. Resolution-wise they are capable of subcellular imaging and fall between the typical medical imaging modalities and the high resolution techniques.

In addition to the usefulness of the green fluorescent protein and other fluorescent markers, recent developments in laser and computer technologies have led to an explosion in the applicability and popularity of light microscopic techniques, especially those based on fluorescence [8, 10-12].

In light microscopy (Figure 1 A), wavelengths of electromagnetic radiation that are visible to the human eye are used to form images. Biological samples such as cells have naturally very little contrast, and they are nearly invisible even when greatly magnified [11, 13]. While there are several useful methods for increasing "naturally occurring" contrast, such as phase-contrast (converts phase shifts to intensity differences) and differential interference contrast (separates polarized light into two components and recombines them) [11, 14], utilizing fluorescence is nowadays the most often used approach [3, 11, 12, 14].

1.1.1. Fluorescence microscopy

Fluorescence microscopy (FM) is based on the use of fluorophores, molecules that emit light of certain wavelengths when excited with light of higher wavelengths. When a fluorophore absorbs a photon, it is excited to a higher energy level, and when the energy level returns to normal, a photon is emitted. Because some energy is lost as heat, the emitted photon has a lower wavelength than the absorbed one. This difference in wavelength is called the Stokes shift, and it enables the detection of fluorescence without interference from the exciting light; the two wavelengths can be separated with a dichromatic mirror. If a fluorophore can be specifically attached to a particular biological target of interest, such as a protein, this target can be visualized with high specificity and excellent contrast against a black background. [12, 13]

There are several ways to make biological samples fluorescent. The two most common methods are GFP fusion proteins (and other fluorescent fusion proteins) and immunofluorescence, both of which have been used also in this thesis. In immunofluorescence, an antibody against the biological target of interest is coupled to a fluorophore. When the antibodies are then introduced to the sample, they function as "light tags" that specifically attach to their targets and make them fluorescent. To amplify the light intensity and to make the labeling easier and more cost efficient, secondary antibodies against the primary antibodies are often used, and these secondary antibodies carry the fluorophore instead of the primary ones. In GFP fusion proteins the biological target protein is directly fused with the green fluorescent protein. GFP fusion proteins can be easily created with recombinant DNA-technology. Countless GFP fusion proteins have been created, and they have been successfully used to image proteins in every major organelle of the cell. GFP has numerous advantages that make it useful in fluorescence imaging: it is non-toxic, creates bright fluorescence efficiently, does not need any co-enzymes or co-factors, is relatively resistant to photobleaching and environmental factors, and remains functional after aldehyde fixatives and can therefore be used also with fixed samples [4]. Indeed, GFP has become a standard tool for measuring gene expression and protein locations. Nowadays there are also numerous variations of GFP, with different

properties such as different excitation and emission spectra. These molecules have revolutionized fluorescence microscopy and become one of the most widely used tools in biomedical laboratories [4, 14].

1.1.2. Fluorescence microscope setups

The most basic setup for fluorescence imaging is a widefield fluorescence microscope (Figure 1 B). In this technique, a filter is first used to select excitation wavelength(s) from light coming from a powerful source, typically a mercury arc lamp. The light is then reflected by a dichroic mirror to the sample, where fluorescence is induced, and the emitted fluorescence travels back to the dichroic mirror, now passing through it and on to another filter, which selects the wavelength(s) to be detected. The formed image can be viewed with an eye-piece or recorded with a camera. The whole sample is illuminated at once, and especially with thick samples out-of-focus light emanating from below or above the focal plane causes blurry images. [12, 14] A more advanced setup for fluorescence imaging is a laser scanning confocal microscope (LSCM), where a point-like laser-beam is used to illuminate the sample (Figure 1 C). The laser beam is moved across the area being imaged (scanning), and the emitted fluorescence is detected with a photo-multiplier tube or a similar device, instead of a camera. The photomultiplier tube converts the analog light signal to digital form, and the digital readout is "synchronized" to the scanning location and speed of the laser beam in such a way, that a digital image is formed pixel-by-pixel. Before reaching the detector, the fluorescent light passes through a very small hole, called the pinhole, which is the central innovation of the LSCM. The pinhole excludes most of the light coming from above or below the focal plane, resulting in much sharper images than with widefield microscopy, and also somewhat improved overall resolution and improved contrast. [14-16] Unlike widefield microscopy, LSCM is inherently a digital technique, and a computer is required to record and display the images created. Confocal microscopes started to become popular in the end of the 1990s and are nowadays considered among the standard workhorses of bioimaging. Despite their popularity, they remain fairly complex machines that require considerable expertise to produce correct and reliable results [15]. There are numerous different types of fluorescence-based microscopes nowadays available, with more being constantly developed. Some of these are discussed in the section "1.1.3 Extensions of the basic premise of fluorescence microscopy".

In addition to excellent and specific contrast, an added advantage of fluorescence is that several different fluorophores can be used simultaneously, because their different excitation and emission spectra allow the signals from each of them to be separated with filters or other techniques. For instance, several different proteins can be labeled with different fluorophores and imaged at the same time. This makes it possible to obtain "multi-color" images to study for instance if different proteins reside in the same locations in a cell at the same time, indicating possible biological interaction. These types of colocalization studies can reveal for instance in which intracellular structure or organelle a certain protein operates, what other proteins it

interacts with, or what receptors a virus uses to get inside a cell [17, 18]. This thesis presents several examples of these types of studies.

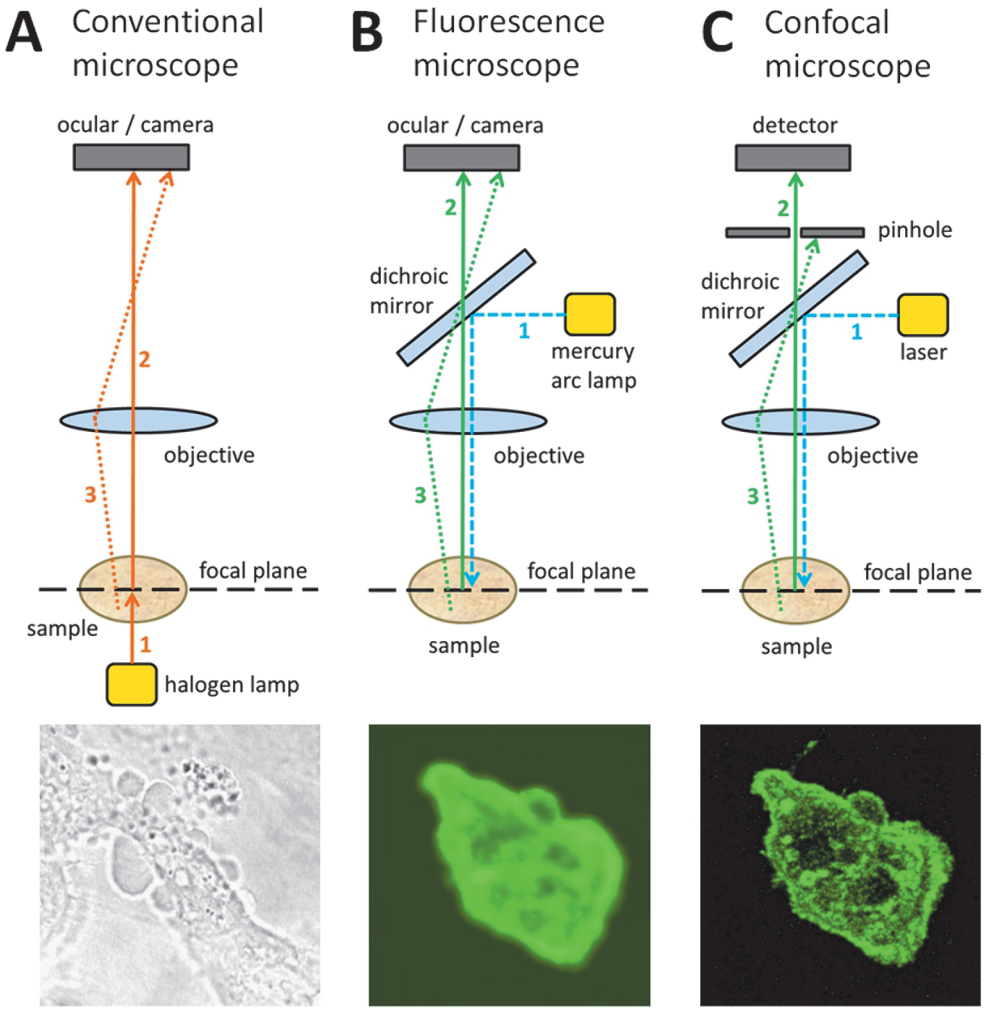

Figure 1. *Typical simplified beam paths of light microscopes.* ***A.*** *In a conventional microscope, the illuminating light (1) goes through the sample, and light from the sample (2) passes directly through to the ocular/camera. Light from above or below the focal plane (3) is also detected.* ***B.*** *In a widefield fluorescence microscope, excitation light (1) is reflected by a dichroic mirror to the sample, and emission light from the sample (2) passes through the dichroic mirror to the ocular/camera. Light from above or below the focal plane (3) is also detected.* ***C.*** *The principle of a laser scanning confocal microscope is otherwise similar to a widefield fluorescence microscope, but in front of the detector is a pinhole, which excludes most of the out-of-focus light (3). Bottom row shows sample images of cells. The fluorescence microscopy image is blurred by out-of-focus light, whereas the confocal microscopy image (maximum intensity projection of a 3D image stack) is sharp throughout.*

1.1.3. Extensions of the basic premise of fluorescence microscopy

Fluorescence microscopy is made more versatile by many "F-techniques", which are variations of the basic premise of using fluorescence in bioimaging [10, 14]. Examples include FRAP, FRET and FLIM. In FRAP (fluorescence recovery after photobleaching) fluorescence is first bleached with high-intensity laser radiation in a selected region, and the recovery of fluorescence to this region is then monitored, yielding information about for instance the connectivity of different cellular compartments or the rate of molecular diffusion or transport [19]. FRET (fluorescence resonance energy transfer) is a phenomenon that occurs if two spectrally overlapping fluorophores are in close proximity to each other and in suitable orientation. Then the fluorophore being excited may transfer the energy of its excited state to the other fluorophore, without releasing a photon itself, and this can be utilized especially to study if two proteins interact, by labeling them with different but "spectrally compatible" fluorophores [14, 20]. In FLIM (fluorescence lifetime imaging), the lifetime of the excited state of a fluorophore is measured, instead of the usual approach of measuring the photon count emitted by this excited state. This enables separation of fluorophores that have highly overlapping spectra but different lifetimes, such as different GFP variants, and it is also an effective way to measure FRET, since the lifetime of the excited state of the "donor" fluorophore decreases when FRET occurs [14, 21].

Fluorescence-based imaging is often supplemented by other contrasting methods [14]. For instance in laser scanning confocal microscopy, a differential interference contrast (DIC) image can be easily obtained at the same time as scanning for fluorescence, and it can provide an overview image of the sample being studied and for instance show where in a cell a particular fluorescence signal is originating from.

The popularity and importance of light-based bioimaging in biomedical research can be illustrated for instance by the usage statistics of the university campus of Turku, Finland, where this thesis has mostly been made. At this campus, services based on light microscopy technologies have been centralized to a Cell Imaging Core facility, the largest of its kind in Finland. In 2012, this facility had users from 83 local research groups, out of the little over 100 research groups in biosciences on the campus in total, and its services were used in approximately 40 peer-reviewed scientific publications. [22, 23] In other words, most research groups used these methods in one form or the other in their scientific work. Numerous recent reviews and other scientific papers also point out the enormous global popularity and importance of these techniques [5-8, 10, 24, 25].

1.2. Multi-dimensional optical sectioning microscopy

In both widefield and confocal fluorescence microscopy, image acquisition can be repeated after changing the plane of focus, and in this way it is possible to acquire cross-section images even through the entire sample, if the image acquisition is repeated a sufficient number of times. While acquiring a single image with these microscopes results in two-dimensional (2D) image data (spatial dimensions X and Y), acquiring a stack of "optical sections" provides information also along the Z dimension, thus resulting in three-dimensional (3D) image data [15, 16]. Computer-controlled microscopes can easily acquire such image stacks automatically. In widefield microscopy, however, out-of-focus light, which makes the sections blurry, greatly reduces the usefulness of the image stacks, and the thicker the sample, the worse the problem. This can be compensated with a mathematical procedure called deconvolution, where the naturally occurring process of convolution in the microscope is calculationally reversed afterwards [14, 26]. Convolution in light microscopy means the process where the image formed from a point-like light source is distorted by the optics and light wave interference phenomena, as described by a mathematical point spread function (PSF). When the PSF is known or can be estimated, it can be used to deconvolve the image data afterwards. However, this process is slow and error-prone, especially if the images are noisy, and the axial resolution (Z) will remain comparatively low [26]. In contrast, in LSCM the pinhole physically removes most of the out-of-focus light, and thus the optical sections created are already clear enough to provide proper 3D information, without the need for any computational post-processing.

In general, 3D-imaging has greatly advanced the field of microscopy [7, 14, 25, 27], for obvious reasons. Everything in biology is three-dimensional, and 2D-data of 3D-samples is easily incorrect or misleading, not only in visualizations but also in quantitative analyses [28, 29]. In addition, 3D-images provide an impressive visual impact that can aid researchers in understanding biological principles on a fundamental level [13, 29]. While widefield imaging has some advantages compared to confocal imaging, such as sometimes lower phototoxicity and considerably cheaper instrumentation [24], deconvolution is necessary for obtaining 3D data, and it is a complex and demanding process, and will give good results only with relatively thin samples [11, 26]. For true 3D imaging, LSCM is in most cases the better choice [15, 24].

1.2.1. Four-dimensional imaging

As the sectioning in 3D confocal microscopy is optical and non-invasive, it can be applied to living samples. 3D image stacks can be acquired at regular time intervals for instance from living cells under physiological conditions, and imaging can go on for hours or even days. This is called four-dimensional (4D) imaging, where time is the fourth dimension, in addition to the three spatial dimensions X, Y and Z (Figure 2).

Obviously everything in biology takes place in a four-dimensional world, and being able to study phenomena in four dimensions is a clear asset and even necessity [7, 13, 14, 18, 24]. According to a popular analogy, using three-dimensional images of fixed cells to determine how the cells work is like trying to find out the rules of a football game from a series of still photographs of a match. It is therefore not surprising that in recent scientific literature, the use of time-lapse imaging has risen exponentially, and covers most areas of the natural sciences [24]. Interestingly, this means that after decades of focusing more on fixed samples, microscopy is going back to where it started from, the direct observation of the dynamics of life [13].

Although very useful, fluorescence microscopy techniques have certain problems. Among the most common challenges are bleaching or quenching of fluorescence caused by the excitation light, and toxic effects of light (phototoxicity) to the biological sample being studied [12]. These problems become more pronounced with 4D time-lapse imaging, where samples are subjected to prolonged or repeated exposure to light. Indeed, live-cell imaging can still today be considered a difficult art: there is no single solution for how to do it correctly, and countless parameters need to be understood and adjusted for acceptable results [24]. Live-cell imaging is always a compromise between image quality, image acquisition speed and phototoxicity, and the key is to find this compromise, which can be different for every experiment [30].

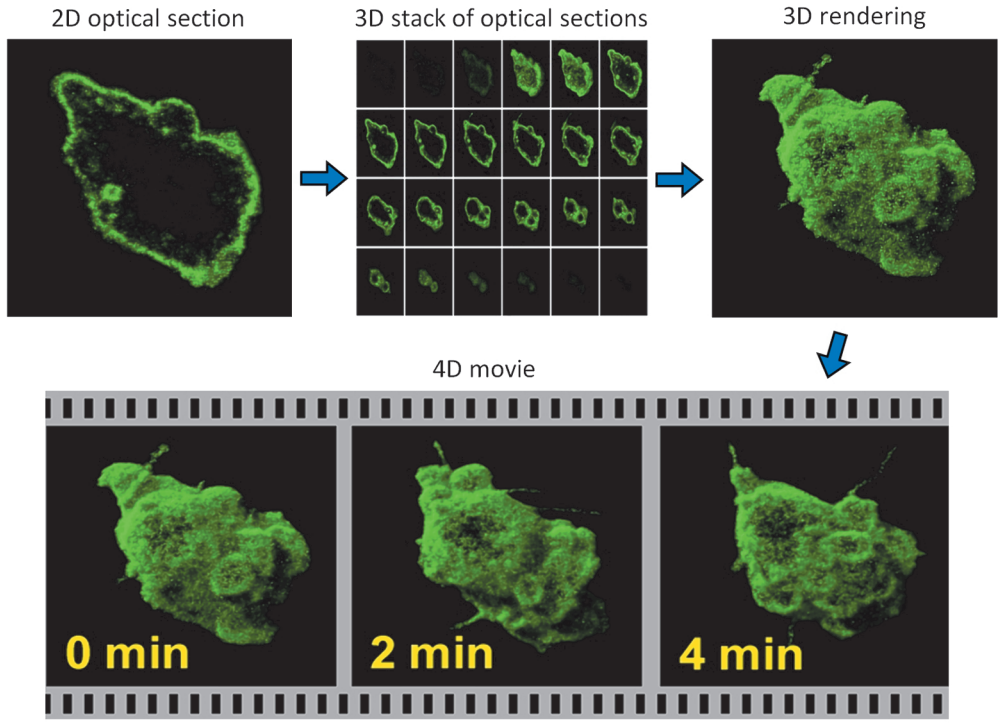

Figure 2. *Four-dimensional confocal imaging. A stack of 2D optical sections contains 3D data, and such a stack can be visualized as a 3D rendering. Multiple stacks can be acquired over time from living samples, each rendered, and the renderings assembled into a "four-dimensional" movie.*

1.2.2. Extensions of the basic premise of optical sectioning microscopy

Multidimensional optical sectioning microscopy is not limited to widefield fluorescence imaging and LSCM. There is a growing number of additional techniques and variations of the basic principles, tailored for different applications and often increasingly complex [13]. A typical problem with LSCM is that the pinhole discards a lot of photons, making the method inefficient and therefore rather slow, especially when considering also the time required for the scanning movement [15]. In spinning-disk confocal microscopy, several pinholes, arranged onto a disk that spins, are used simultaneously to form the image, which can be recorded with a camera. This increases acquisition speed, but sensitivity and adjustability are more limited than with LSCM [15].

Another problem with LSCM is that its penetration depth is not particularly good, and while sufficient for single cells, it is often insufficient for multi-cellular samples, tissue sections, small model organisms, or *in vivo* applications. The penetration depth is improved by two-photon laser scanning fluorescence microscopy, where two successive low-energy photons with longer wavelengths are used to excite fluorophores, instead of the single shorter-wavelength photon of normal LSCM. Penetration depths of up to 1 mm can be achieved, and an added advantage is that in two-photon excitation photobleaching is limited to the focal plane, unlike in LSCM. [31]

In total internal reflection fluorescence microscopy (TIRFM), fluorescence is excited by a narrow field of light just above the glass surface on which the sample is placed. This light field is called an evanescent field, it is only a few hundred nanometers thick, and it is created by a laser beam targeted to the opposite side of the glass at such an angle, that total internal reflection occurs [32]. This method has high axial resolution and is well suited for single molecule imaging, but only one optical section near the glass surface can be imaged. 3D information can still be obtained in some cases by observing fluorescence intensity, but the technique has many limitations in for instance motion tracking applications [33].

Modifications to the widefield imaging approach have also been developed, one example being structured illumination microscopy (SIM). In this technique several images are acquired with sinusoidal illumination patterns that are slightly shifted between the acquisitions, and the final image is calculated from a combination of all of the images. This method can effectively reduce out-of-focus light without deconvolution, and achieve an axial resolution approaching that of the LSCM, with less costly instrumentation. Under some circumstances, the resolution of SIM can even exceed that of the LSCM, both laterally and axially, but LSCM has better penetration depth. [15, 34]

1.2.3. Super-resolution techniques

A major limitation of all the techniques mentioned above, and indeed of any light-based imaging technique, is the wavelength of light, which limits the resolution to about 150-200 nanometers [35]. Sub-cellular research would often need higher resolution, and it has even been suggested that most cell-biological questions could be answered, if it was possible to non-destructively image specific molecules in real time with the resolution of an electron microscope [36]. Fluorescence microscopy techniques can provide all the other qualities, except the resolution. However, recently numerous super-resolution techniques have become available, in an attempt to overcome this problem [25, 35]. Some of the most well-known examples are STED, STORM and PALM [35]. All of these are based on switching fluorophores on and off sequentially, and there are two main approaches for doing this. In the targeted mode a spatial light distribution is used to switch off fluorophores in such a way that only a spot with sub-diffraction dimensions remains "on". In stimulated emission depletion microscopy (STED) this is achieved with a doughnut-shaped distribution that leaves on only the fluorophores in the center of the doughnut, creating a light spot smaller than possible with conventional LSCM and thus increasing resolution. In the stochastic switching mode, individual molecules are randomly switched on in space, and the locations of these molecules are calculated from their centroids. This approach is utilized in photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), both of which are widefield techniques, unlike STED, which is a scanning technique. The stochastic methods have the advantage that each fluorophore only needs to be switched on once, and they are applicable especially in TIRFM. However, both image acquisition and the successive calculation are time consuming, whereas targeted approaches can produce images faster and without needing post-acquisition calculations. Super-resolution techniques require special fluorophores, but they can achieve lateral (X and Y) resolutions of up to few tens on nanometers. Most of the techniques do not improve axial (Z) resolution, but there are some exceptions, such as 4Pi microscopy, where two opposing objective lenses are used to improve axial resolution up to sevenfold [35], and a phasor-based method that can produce axial resolutions close to 10 nm with a conventional confocal microscope [37].

Despite improvements in resolution offered by the super-resolution techniques, the highly important fourth dimension, time, remains a problem. Compared to conventional confocal microscopy, super-resolution techniques require longer acquisition times to obtain the additional spatial information [35, 38]. Of other high resolution techniques, atomic force microscopy is also slow, and in addition limited to surface topographical imaging, and electron microscopy is not suitable for time-lapse imaging at all. Improvements are being developed, such as a recently reported faster STORM based on compressed sensing [38], but so far conventional confocal microscopy techniques remain the most widely used methods for 4D bioimaging [15]. 4D laser scanning confocal microscopy is also the principal bioimaging technique used in this thesis (although the use of atomic force microscopy is also demonstrated).

1.3. Bioimage informatics

Bioinformatics is now a well-established field of science and plays a fundamental role in biology [27]. Genomic databases, and various interaction networks from genomics, trancriptomics and proteomics are routinely used in biological research in the present era of informational biology [1, 27]. However, cells are highly complicated biological units that interact, communicate, have spatiotemporal sub-compartments and organization, and many different mechanisms that critically affect how they operate and how their genes operate. These factors cannot generally be taken into consideration in standard bioinformatics [1], whereas bioimaging can be used to study them. Historically, microscopy has been a qualitative methodology, although there was some work on quantitative analysis such as segmentation and motion tracking already early on in the development of fluorescence microscopy [2, 18, 39]. In recent years, however, the field of microscopy has begun to experience a major revolution: the development of numerous advanced instruments, computational methods and software have started to turn microscopy into a quantitative science [2, 18, 39]. The visualization, processing and analysis of images produced especially by light microscopic techniques has become its own new field of science, called bioimage informatics [1, 8, 27, 40].

Of particular significance in bioimage informatics are high-throughput and high-content screening applications, which have become routine in bioimaging during recent years, propelled by developments in automated microscopy, robotics and computers [3, 11, 41, 42]. These approaches are effectively used for instance in drug screening, developmental biology and stem cell research [8], but a common problem with them is the need for software solutions for dealing with the very large data amounts produced and the complex analyses required [3, 8, 42]. This is one of the main general challenges of bioimage informatics. On a more detailed level, some of the aspects that bioimage informatics deals with are for instance bioimage file formats and practical software development [27]. Current light microscopes produce data in tens of different image formats, but rapid developments in the field and the different requirements of different imaging techniques make it unfeasible to establish common standards [27]. It is therefore important for bioimage informatics software to be able to read the various file formats directly [27, 40], which requires interoperability and collaboration, and ideally the establishment of common libraries for file format reading, such as Bio-Formats [40]. These needs are obviously reflected in the practical software development of the field, where they imply that community-driven open software development projects are the best approach, and that different software products need to be able to interact with each other, share information and tool libraries, and at the same time provide advanced tools for analysis, visualization and processing in a practical and organized manner [27]. These are not easy requirements to fulfill.

It is perhaps interesting to note that bioimage computing approaches are needed not only in analyzing bioimages, but they are often also a necessary pre-processing step

to improve quality or throughput for instance in many of the super-resolution techniques [40]. Bioimage computing also paves the way for obtaining mathematical models for the various dynamic structures and processes of cells [18]. Bioimage informatics is a multidisciplinary field, where computer scientists, physicists and biologists alike can become proficient, but they need to work seamlessly together to obtain good results [1, 24, 43]. This thesis is a fitting example of work in the field of bioimage informatics, although the term was not even in use yet when the work was started.

In bioimage informatics various databases are also increasingly being developed for different model organisms, the Allen Brain Atlas database being one example [27, 40]. Approaches are also being developed for searching data from such databases using sophisticated methods, such as retrieving images that resemble a query image based on some numerically calculated features, as in the recently reported OMERO.searcher [44]. While storing image data into such databases and retrieving and sharing it from them will be important in the future development of bioimage informatics [5, 6, 27, 44], this thesis focuses only on the image processing, analysis and visualization aspects of bioimage informatics.

Bioimage informatics is still a very new field, but it is likely to become elemental in the era of informational biology, in deciphering how genes, molecules and cells actually work and interact [1, 2, 8]. It has been pointed out that there are similarities between the current state of bioimage informatics and bioinformatics, when it was in its early stages in the early 1980s [1]. A similar explosion in popularity and significance as happened with bioinformatics is likely to happen also with bioimage informatics [27], but currently the field is still somewhat chaotic, and successful software development will be crucial in order for the field to live up to its potential [1, 3, 8, 42]. The future of bioimage informatics is discussed further in the chapter "7. Conclusion and the future of bioimage informatics".

1.4. Bioimage informatics software

In modern bioimaging, computer software is needed to control advanced imaging devices such as confocal microscopes. Microscope manufacturers usually supply their instruments with software that is good enough for image acquisition, but limited in post-processing capabilities [6, 41]. Yet, when the images have been acquired, only half of the job is done. It is the post-processing of the image material that is needed to extract useful visualizations and quantitative results, especially in the new era of bioimage informatics. While the key components of bioimage informatics are often defined to be the visualization, processing and analysis of bioimages [1, 8, 27, 40], it is these same components that are also defined as the biggest problems in the field: there is simply a lack of software tools to carry out these tasks [3, 8, 10, 25, 27, 42]. When this thesis work was started, there was hardly any advanced bioimaging software available [1], and programs that were available were often lacking critical

features such as the ability to properly handle 4D image data. Among software that was available were AxioVision from Carl Zeiss and Imaris from Bitplane, both of which are still in use today. Despite these, it took a lot of work and innovation, and combining several different computer programs, to create the images and videos in publication I, and quantification of the phenomena visualized was not seen as particularly realistic at the time.

Of course, for decades there have been software and algorithms available for image processing in general (for example Adobe Photoshop or Corel PhotoPaint), and for quite some time the software situation has also been better for medical imaging than for light and electron microscopy. While these software packages might be useful in biological microscopy in some situations, typical biological microscopy data is very different from other types of image data, even from typical medical imaging data: biological microscopy images are often very rich in information content and dynamic complexity, and having different dimensions, signal-to-noise ratios and specific labeling (fluorescence data channels) than other types of images. Together with the fact that biological microscopy often requires new types of analyses to be developed, these differences dictate that software tailored specifically for biological microscopy is required in most cases [5, 40, 43]. The realization that such software is needed has been somewhat slow in the biomedical research community, perhaps because it was long thought that image acquisition and simple visual inspection of the images was sufficient. Nowadays it is frequently recognized that bioimaging requires these specialized software tools, even just for visualizing 3D-data [3, 8, 10, 38, 40-42], and that their development has been lagging behind [8, 10, 25, 27]. Today, some in the community even consider bioimaging software development as a highly important area of biological research [2]. It has also been speculated that bioimaging software might revolutionize microscopy, by providing substantial gains in time, reproducibility and objectivity, and by enabling extraction and further use of the enormous information content of bioimages [42].

1.4.1. Features of bioimage post-processing software

The features required of bioimage post-processing software vary depending on the application, but there are some general features that are needed or useful in most cases, such as the following.

Visualization. Specialized visualization tools are required for viewing especially the multi-dimensional data typically produced by modern imaging devices. Visualization tools are also required for checking and adjusting the operation of quantitative analysis tools. In addition to various 2D visualization modes, such as viewing sets of optical sections individually or viewing cross-sections of 3D datasets, there are several 3D rendering methods for creating truly three-dimensional representations of 3D-data. These can be divided into two main classes: volume and surface rendering. In volume rendering translucent representations of entire image volumes are created by for instance ray casting, whereas in surface rendering surfaces of structures defined by for instance iso-intensity values are visualized using geometric primitives such as

small triangles. With 3D/4D data, movies or animations also often need to be created, showing 3D renderings rotating, panning and zooming, or showing changes over time. [6, 10, 18, 30, 40]

Image processing. One of the most typical image processing needs with fluorescence-based images is to reduce noise caused by fluctuations in the illumination or thermal fluctuations in the detector, and to reduce background signal caused by for instance autofluorescence [30]. Filters such as median filter or anisotropic diffusion are frequently used. Another typical image processing need is to adjust the intensity transfer function (ITF), for instance to increase image brightness or contrast. The graph of the intensity transfer function should be shown, as many scientific journals have instructions for its acceptable shape [45]. Image processing tools also include for instance common arithmetic operators, such as addition and subtraction, and logical operators, such as "and" and "or". Other commonly used image processing algorithms calculate e.g. deconvolution, where image quality is enhanced by mathematically reducing the effects of light scattering [14, 26], and registration, where several similar images or several timepoints are aligned with each other to make them comparable [18, 39].

Pixel/voxel-based analyses. These are quantitative analyses that are carried out to the entire image data (or some simple and often manually defined sub-region of it), pixel-by-pixel or voxel-by-voxel. A typical example is the calculation of average or total fluorescence intensity, for instance to measure fluorophore concentrations [30]. Very popular are also numerous colocalization analysis methods, which study whether two fluorescent markers have the same spatiotemporal location (Figure 3 A) [17, 46, 47].

Segmentation-based-analyses. Segmentation means separating the objects of interest from the rest of the image data (the background). After segmentation, the objects can be classified and analyzed for a variety of parameters, such as number, size, shape and distribution (Figure 3 B). Segmentation opens up numerous possibilities for complex analyses, such as object-based colocalization or motion tracking, where the same segmented objects are identified in successive timepoints, and parameters such as speed and direction of movement calculated. There are numerous segmentation methods available, the most common probably being thresholding-based approaches, where one or several intensity values, determined either automatically or manually, are used to discriminate between objects and background. Other common approaches are edge-based methods (e.g. watershed segmentation) and region-based methods (e.g. region growing segmentation). [18, 30, 39, 40]

Annotations and storage. In addition to the more obvious feature requirements listed above, also the closely related aspects of image annotation and storage should be considered. Annotating means associating images with information about them, such as acquisition settings and sample descriptions. This type of information is called metadata. With constantly increasing data amounts, going up to tens of terabytes per day [1], storing the images is also no longer self-evident or simple, and requires

database software solutions. Annotations are used to manage and query the stored images, and eventually also atlases can be assembled. [6, 39, 40]

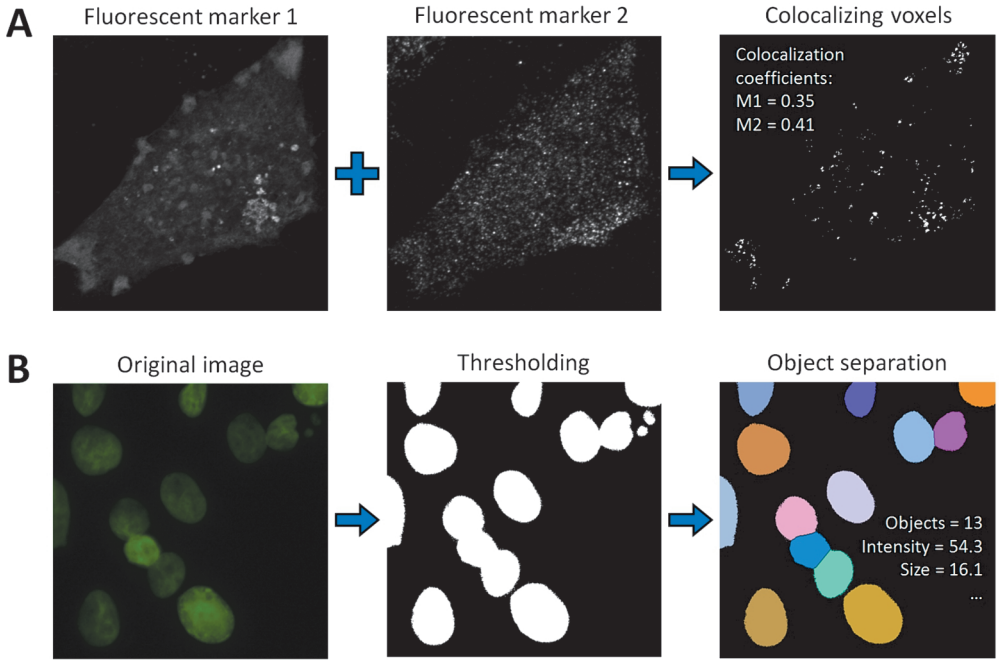

Figure 3. *Quantitative image analysis.* ***A.*** *Colocalization analysis is an example of pixel/voxel-based analysis methods. Here, images from two fluorescent markers are first shown separately. The "colocalization map" on the right is a 1-bit representation of all voxels that exceed certain intensity thresholds for both of the fluorescent markers. These colocalizing voxels are analyzed quantitatively for instance by calculating Manders' colocalization coefficients, which indicate colocalization as values between 0 and 1* [46]. ***B.*** *In segmentation-based analyses, an image is first divided into "foreground" and "background" for instance by thresholding, and the foreground is then separated into different objects based on criteria such as connectivity. The objects are labeled and often colored with arbitrary colors that assist in their visual inspection. Segmented objects can be quantitatively analyzed for numerous parameters, such as number, intensity and size. All images are maximum intensity projections of 3D datasets.*

1.4.2. Available software packages

During the last ten years or so, numerous software options have become available for bioimage post-processing [6, 39, 40]. These include, but are not limited to, the following:

Proprietary software. These are commercial software packages with closed source code. The best known examples include Imaris (Bitplane) and Volocity (PerkinElmer), which are large and versatile packages for both visualization and analysis of

multidimensional images. Other examples are Huygens (Scientific Volume Imaging), known especially for its deconvolution features, and microscope control programs and their extensions, such as ZEN (Carl Zeiss) and Fluoview (Olympus). These contain post-processing tools to a varying degree, and are often more limited than software specifically designed for versatile post-processing [6].

Open source software. An increasing number of bioimage software tools have open source code, which means that the code is freely available and modifiable to anyone. This allows for better transparency, reliability and reproducibility of science than proprietary software [6, 43]. Probably the best known and most widely used example is ImageJ [48], which has been developed for more than 25 years, and operates primarily on single 2D images. Fiji [49] is a popular modernization of ImageJ, offering improved architecture and multidimensionality support. Icy [50] is a new 3D visualization and analysis software package with graphical processing pipelines. Vaa3D [51] also supports 3D data, but is a primarily visualization-based tool. BioImage Suite [52] is a slightly older image analysis package, primarily for medical and neuroimaging applications. CellProfiler [53, 54] is designed for high-throughput screening and phenotyping of cellular images, and pyBioImage [55] is an example of even more specialized software, developed for immunological research and imaging of germinal centers. Many open source projects increasingly support interoperability, for instance Icy and CellProfiler can connect to ImageJ. The Open Microscopy Environment (OME) [27] is a large project focusing specifically on the interoperability between image data visualization, analysis and storage applications. OME and its several extensions, such as OMERO, are especially aimed at managing microscopy databases [6, 44].

Programming tools. In addition to the easy-to-use software packages listed above, targeted towards the average end user, there are also other tools suitable for bioimage post-processing, based on the philosophy that the user understands programming. This offers substantial flexibility, but only to a limited number of users with the necessary skills. Examples include LabVIEW (National Instruments), a visual programming language platform especially for instrument control, KNIME [56, 57], an open source modular environment for visual assembly of data pipelines and algorithms, and MATLAB (Mathworks), a programming environment for numerical computing, optionally equipped with an image processing toolbox.

Bioimaging libraries. There are several open source tool libraries available for image processing, analysis and visualization [6]. These libraries are usually not used directly by the end user, instead they are used through some of the many bioimaging software packages that utilize them. The Visualization Toolkit (VTK) [58] is one the best know examples, focusing on interactive 3D visualizations with multiple rendering methods. The Insight Segmentation and Registration Toolkit (ITK) [59] is often used together with VTK, and it focuses more on processing and analysis than visualization. For instance Vaa3D uses both VTK and ITK, and Icy and pyBioImage use VTK. Bio-formats [40] is a Java library of file readers, enabling direct reading of all popular microscopy file formats, and utilized by software such as ImageJ, Fiji and Icy.

1.4.3. Requirements for bioimage informatics software

Despite the various available software options listed above, lack of software is still often considered a problem. One reason for this is the rapid increase in high-throughput applications, and other requirements of bioimage informatics, such as handling large multi-dimensional files, which most of the current software solutions have not exactly been designed for [1, 25, 27, 41, 42]. There are also numerous general criteria that bioimaging software should fulfill. Carpenter, Kamentsky and Eliceiri recently assembled a list of such general criteria, according to which bioimaging software should be for instance user-friendly, intuitive, support a wide range of applications, be multi-platform, support reproducible scientific research, be extensible with modular architecture and defined interfaces, have open source code and be developer-friendly, have organized software design, be validated and functional, scalable, robust, and tested for actual biological applications [5]. Many other authors have brought up similar criteria in different contexts, emphasizing especially aspects such as the need for open source code for transparent and reproducible science [5, 10, 39, 40, 43]. Finding software that fulfills these general requirements, has the features specifically needed for bioimaging, and is suitable for modern bioimage informatics, was impossible when this thesis work was started, and it is still very difficult today. It was therefore natural that bioimaging software development became a major part of this thesis.

1.5. Biological case study: α2β1 integrin

When developing scientific research tools, irrespective of what they are, practical applications should ideally be guiding the development, and the tools being developed should be frequently tested in actual research situations [27]. In this thesis work, human integrin α2β1, a cellular collagen receptor and signaling molecule, was used as a case study when developing 4D confocal imaging approaches and image processing software. The practical applications in all of the original publications I-III are centered on this molecule, and all the tools were initially developed from this point of view.

It is important to note that even though the methods developed in this thesis were carried out with integrin case studies, they were developed to be general purpose tools, and are widely applicable. It should also be noted that many other tools were also developed in this thesis, not only those used for integrin studies.

1.5.1. The integrin family

Integrins are an extensive family of cell adhesion and signaling receptors. Structurally they are transmembrane heterodimers, consisting of non-covalently linked α and β subunits, and 24 different subunit combinations are known to exist in humans. Integrins anchor cells to their surroundings, both to other cells and to the extra-cellular matrix, and they are also important mediators of signaling both into cells and

out of them. Integrins are involved in several important physiological and pathological processes, such as development, the immune system and cancer. [60, 61] Intracellularly they are often linked to the actin cytoskeleton [62].

Integrins have different structural conformations (Figure 4). Typically, they are bent when inactive, and extended when active, although also the inactive conformations may bind ligands. Inside-out signaling of integrins possibly "switches" them between the active and inactive conformations, whereas outside-in signaling is initiated by ligand binding and leads to conformational changes that trigger complex and varied signaling cascades. The different conformations of integrins are involved in the precise and very dynamic regulation of their activity, in addition to integrins being regulated by transcriptional and locational mechanisms. [63, 64]

Integrins are very common proteins, and they are expressed in all eukaryotic cells. Integrins often operate in complex biological processes that involve also many other molecules and possibly also other integrins, and many of their functions are still unknown. [60, 61]

α2β1 integrin is one of four collagen receptors in humans (Figure 5) [65]. The ligand recognition and binding site of the α2β1 integrin is a specific domain in its α subunit, called the I-domain. All collagen receptor integrins, as well as five other integrins, have this domain [60], which has two structural conformations, "open" and "closed", and the open conformation typically has a higher affinity for ligands [66]. The conformations of the I-domain are linked to the conformations of the whole integrin molecule. α2β1 integrin makes an interesting case study for developing bioimaging tools, for the reasons that are discussed in the sections below.

1.5.2. Cluster formation and lateral movement

Normally α2β1 integrin is located on the cell surface, as a trans-membrane protein, and more or less evenly distributed on the cell membrane [67]. This gives it a clearly defined cellular location, which is an advantageous starting point in imaging applications. Because of its extracellular domains, the integrin is also easy to label with fluorophores without needing to get dyes through the cell membrane. For instance immunofluorescence can directly be used, even with living cells, as has been done in this thesis. The extracellular domains also make it easy to "interact" with the integrin from the outside, for instance by introducing agents such as ligands that induce some behavior in the integrin. In this thesis, for instance secondary antibodies have been used in this fashion.

α2β1 integrin is likely to move laterally on the cell membrane [67-69], which makes it dynamic and therefore at the same time challenging and interesting from the point of view of bioimaging. The integrin provides possibilities for motion tracking that are at the same time more demanding than tracking for instance the movements of entire cells, but also perhaps more clearly defined than the typical more random and "axial" movements of endocytic vesicles. The lateral movements of the integrin may be

involved in processes such as focal adhesion formation and collagen fibril remodeling [67, 70].

As integrins in general, and also many other receptor molecules, α2β1 integrin forms clusters on the cell membrane as part of its normal activity (Figure 5) [61, 67, 71, 72], for instance when binding multivalent ligands such as collagen fibrils [73]. The clustering is probably related to the lateral movement: by moving on the cell membrane integrins are able to join each other and form the clusters. The clustering, likely controlled through interactions between the cytoplasmic tails of the integrin subunits, plays a role in regulating integrin activity in addition to the different conformations of the individual molecules [61, 67, 68]. Clustering may also be a mechanism that explains how a single receptor can have so many different roles: It has been suggested that because integrin binding to a monovalent ligand does not induce clustering, it leads to cell adhesion via existing focal adhesions, whereas integrin binding to a multivalent ligand, which also induces clustering, leads to cytoskeletal re-organization via the formation of new focal adhesions. Clustering by non-ligand aggregators (such as non-inhibiting antibodies), in contrast, would then lead to signal transduction only. [71]

When molecules cluster together, their appearance in microscopy images changes in terms of parameters such as size, shape and intensity. This makes their quantitative analysis from images challenging, but if it can be done, the protocols developed will be robust and widely applicable.

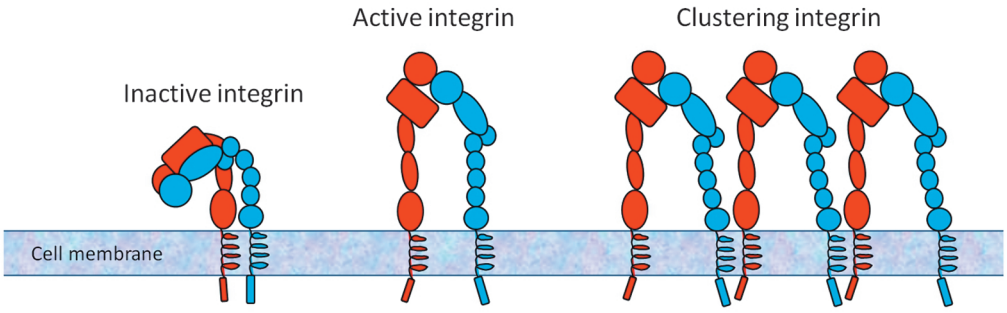

Figure 4. *Schematic representation of the probable inactive (bent) and active (straight) conformations of α2β1 integrin, and an illustration of several integrins clustering together on the cell membrane. The α subunit is colored red and the β subunit is colored blue.*

1.5.3. Receptor of Echovirus 1

Some natural pathogens, such as human Echovirus 1 (EV1), use α2β1 integrin as their cellular receptor [74]. Echovirus 1 belongs to picornaviruses and can cause respiratory and other infections or even fatal neonatal illness [75]. Many other picornaviruses also use integrins as their receptors, for instance coxsackievirus A9 uses αV integrins

[76], but Echovirus 1 would seem to use α2β1 specifically, and the entry process is also different from that detected with other integrin-binding viruses [76, 77].

α2β1 integrin being the receptor for EV1 widens the applicability of integrin research tools to virus studies, and enables viruses to be used as models to study the functions of the integrin. Furthermore, the internalization process of the virus can be used as a model to develop artificial particles that mimic the virus and can specifically enter cells and for instance carry therapeutic payloads. This type of work was successfully carried out in publication III of this thesis.

1.5.4. Internalization

Although normally located on the cell membrane, α2β1 integrin can be internalized into the cell as part of its normal biological activity. This internalization can be initiated for instance by clustering the integrin with antibodies, or by the above-mentioned Echovirus 1 [77]. In many biomedical studies, the internalization of molecules into a cell is of interest, for instance in drug development. α2β1 integrin can therefore be used as an effective model for developing tools to study cellular internalization, as illustrated also by the work in this thesis. As mentioned, the integrin can be easily fluorescently labeled from the outside when it is still located on the cell membrane, and its internalization can thereafter be initiated and followed by fluorescence-based microscopy methods, as has been done in this thesis. When the fluorescent staining is done with a dye that does not spontaneously go through the cell membrane, there will be no fluorescence inside the cell in the beginning of the experiment (as there would be if for instance a GFP fusion protein was used), which makes following and quantifying the subsequent internalization much easier.

α2β1 integrin is particularly interesting also because its internalization does not seem to involve the classical clathrin-mediated pathway, but seems instead to be dependent on for instance caveolin-1 and protein kinase C [77]. There are indications that the entry would take place via a macropinocytosis-like mechanism, after which the integrin would be targeted to caveolin-1-positive multivesicular bodies [76, 78]. Interestingly, collagen receptor integrins normally recycle between the cell membrane and endosomes, but α2β1 integrin was recently reported to enter a non-recycling calpain-dependent degradative pathway after internalization induced by clustering [79]. In other words, both the internalization mechanism and the subsequent fate of the internalized proteins seem to be rather unique in the case of the α2β1 integrin, supported also by the fact that Echovirus 1 has not been observed from other intracellular organelles, such as Golgi or the endoplasmic reticulum [80].

1.5.5. Dynamic activity

α2β1 integrin is very dynamic, but the movements it undergoes are likely to be neither too fast nor too slow for typical 4D LSCM imaging setups. Whether moving laterally on the cell membrane or internalizing, the integrin clusters are rather large and stable and involved in fairly slow processes such as focal adhesion formation and

cell migration [81, 82], and might therefore be expected to move in a fashion that suggest slow directed motion rather than faster Brownian-type motion. Another dynamic event related to α2β1 integrin, the entry of Echovirus 1, is also a relatively slow process [77, 78]. Thus, α2β1 integrin should be a good subject for motion tracking applications. (The dynamics of α2β1 integrin are discussed further in the section "6.3.3. Motion tracking".)

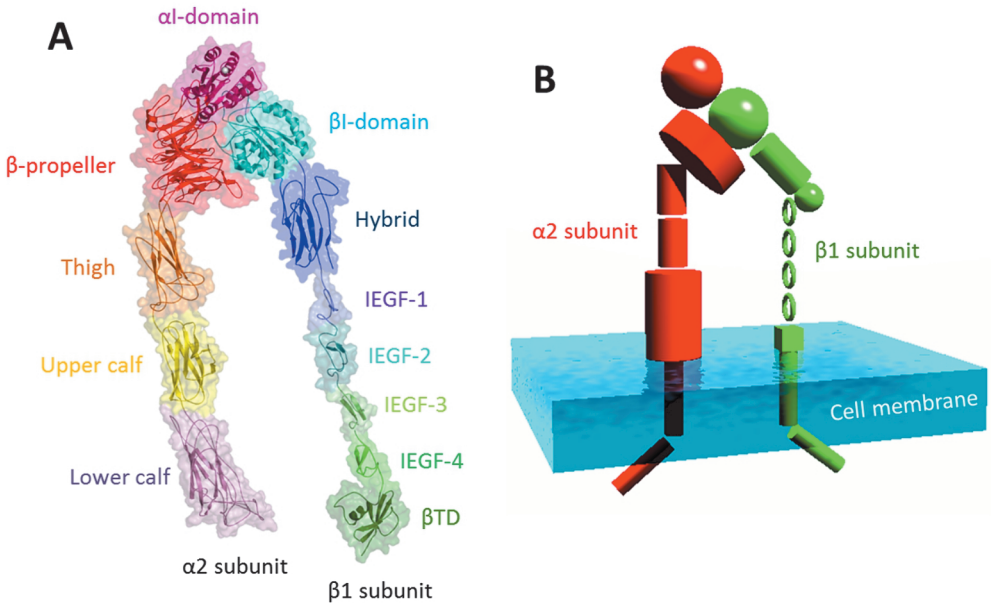

Figure 5. *Human α2β1 integrin.* ***A.*** *Molecular model of the ectodomain of the integrin, showing the different domains of the two subunits, including the ligand-binding I-doman of the α subunit.* ***B.*** *Schematic representation of the integrin on the cell membrane. The α subunit is colored red and the β subunit is colored green. Molecular models created by Daniel White* [65].

1.5.6. Biological significance

In general, integrins are biologically very important molecules [61]. α2β1 integrin is a central mediator of cell adhesion, which is a critical feature of all multicellular organisms, and integrins are found in all multicellular life. It has even been suggested that integrins have been involved in the development of multicellular life [83]. α2β1 integrin is primarily a receptor for fibrillar collagens, which are the most abundant proteins in the human body, but it is also the receptor for basement membrane collagens, and for instance several laminins [66], tenascin [84], the cartilage protein chondroadherin [85], proteoglycans such as decorin [86] and perlecan [87], and matrix metalloprotease 1 [88], an enzyme involved in the breakdown of the extracellular matrix. Different collagen receptor integrins are expressed in different cell types, and α2β1 integrin is expressed primarily in fibroblasts, platelets and epithelial and endothelial cells. As suggested by its ligands and patterns of expression,

α2β1 integrin is involved not only in the maintenance of multicellular organization, but also has many other important biological roles.

Fibroblasts remodel collagen, and this phenomenon is essential in development, wound repair and regulation of cell growth. α2β1 integrin has been implicated in all these processes, and may be the receptor through which collagen fibril formation occurs [70]. α2β1 integrin has also been discovered to control cell cycle progression through $p27^{KIP}$ [89], and cell migration through p38 MAPK signaling [90].

α2β1 integrin operates in the immune system. It is expressed for instance on activated T cells, and inhibiting it prevents inflammatory responses [91]. On platelets, α2β1 integrin is critical in initiating blood clotting. The integrin may bind its ligands also in inactive conformation, and the ligand binding may then induce an active conformation, strengthening the binding. This may be part of the mechanism how platelets initially recognize collagen in a wound, and then bind more strongly through the activation of the integrin [92].

α2β1 integrin also plays a role in numerous pathological conditions. Abnormal α2β1 expression has been detected in several cancers. For instance invasive melanoma cells over-express α2β1 [93], whereas several other cancer types, such as breast and colon carcinomas, show reduced expression [94, 95]. Other diseases where α2β1 integrin has been implicated include multiple sclerosis [96], psoriasis [97], atherosclerotic plaque formation [98] and even some bacterial infections [99]. New roles for α2β1 integrin in significant pathological conditions also seem to be frequently discovered. For instance, recently α2β1 integrin was found to be a necessary component of lethal prostate cancer metastasis [100], to possibly be a receptor for Immunoglobulin A in the common kidney inflammation IgA nephropathy [101], and to have a role in the second most common rheumatic disease, Sjögren's syndrome [102].

Despite numerous indications of the significant biological functions of α2β1 integrin, knock-out mice have displayed surprisingly mild phenotypes: they develop normally and are fertile [103, 104]. These mild phenotypes may be explained by the fact that there are several collagen receptor integrins, and if one of them is unavailable, others might be able to take over the most crucial functions. It should also be noted, that while integrins in general are ancient constituents of multicellular life, collagen receptor integrins are a more specialized sub-group of integrins that have develop later in evolution and are present only in vertebrates [105]. They may therefore not be so critical to development, but still necessary in specific situations and processes. In any case, α2β1 integrin knock-out models do display distinctive phenotypes, and especially more recent models have confirmed some of the proposed roles of the integrin in for instance certain cancers and in the functioning of platelets [106, 107].

All the various biological roles of α2β1 integrin make it biologically interesting also in itself. Deciphering the mechanisms of its functions can help in understanding important biological processes, and aid in the development of treatments for several

pathological conditions. Furthermore, it is sensible to develop bioimaging tools with a case study that has actual biological significance and potentially important implications. Not surprisingly, around the time when this thesis work was started, it was pointed out by Richard Hynes, one of the key authorities on integrins, that bioimaging is likely to be of significance in integrin research in the coming years [61].

2. Aims

This thesis is cross-disciplinary, spanning computer science, software engineering, cell biology, bioimaging, bioimage informatics and nanoscience. The aims of the thesis form a continuum, from imaging to image processing to practical applications. A common denominator is the biological case study, α2β1 integrin, which played a central role at each step of the continuum. Another common denominator is the main underlying aim of the whole thesis, to promote advanced bioimaging (aim 4). The main aims can be summarized as follows:

1. Develop protocols for modern 4D imaging of cell surface receptors, making it possible to clearly see the molecules move laterally, cluster together and internalize, all in a living cell under normal physiological conditions. (When this thesis work was started, hardly any such imaging had been done, and 4D confocal microscopy was just taking its first steps in Northern Europe. There were no existing protocols or software tools available for making advanced visualizations and videos.)

2. Develop scientific software for visualizing, processing and quantitatively analyzing bioimaging data. The software should be capable of 4D animations and quantitative analyses of cell surface receptor clustering, internalization, colocalization with other cellular markers and motion tracking, but the software should also be a general-purpose tool, widely applicable in biomedical imaging and freely available to anyone. (When this thesis work was started, post-processing 4D bioimaging data was a major bottle-neck in bioimaging, due to lack of suitable software.)

3. Apply the techniques and tools developed to something more practical in a closely related field of science, to validate the methods further and demonstrate their wide applicability. The application could be artificial nanoparticles developed to mimic the internalization process of the natural cell surface receptors studied, aimed to deliver payloads to specific cells and specific cellular compartments. The application should demonstrate possibilities to quantitatively study the trafficking and internalization of any particles in cells and to include the use of additional bioimaging methods, such as atomic force microscopy.

4. Promote the use and development of advanced bioimaging in biomedical research on an international level. This can be done for instance by producing tools and protocols that are available to everyone and realistically applicable to many different bioimaging tasks, and by disseminating information about advanced bioimaging. (When this thesis work was started, the importance of what would later be called bioimage informatics was predicted, but at the time the biological research community was still largely lacking in appropriate information and tools.)

3. Materials and Methods

The materials and methods used in this thesis are described in detail in the original publications I-III. This chapter gives an overview of the most important materials and methods used.

Cells, cultures and sample preparations. Human osteosarcoma cells (SAOS) were the main cells used in the experiments, in particular a cell line stably transfected to express α2β1 integrin. The FuGENE reagent was used for transfections of GFP constructs. Integrin clustering was induced by first incubating the samples with a non-function-blocking mouse antibody against α2β1 integrin, and then introducing a secondary anti-mouse antibody, which was also coupled to a fluorophore. In some cases, integrin clustering was induced by incubating the sample with Echovirus 1. Alexa dyes were used as fluorophores in most cases, and in some cases CellTracker was used to stain the entire cell volume. Prior to imaging, cells were grown at 37 degrees Celsius either on high quality cover glasses (for fixed samples) or on chambered cover glasses (for living samples). For fixed samples, cells were fixed with paraformaldehyde at room temperature and permeabilized with triton X-100. Additional labeling was done with immunofluorescence as needed, and cells were mounted with Mowiol-Dabco.

Nanoparticles. Stöber silica particles were used. The particles were typically coated with polyethylene glycol and a streptavidin-Alexa dye conjugate, and different address labels were created by incubating the particles with different biotinylated antibodies. Dynamic light scattering and electron microscopy were used in quality control.

Confocal microscopy imaging. Confocal imaging was done with Carl Zeiss microscopes, mainly an Axio Observer.Z1 with LSM 510 and an Axiovert 100M with LSM510. A 63x 1.4 oil immersion objective was used in most cases, and fluorescence excitation and emission detection were carried out separately for each excitation wavelength, with light path settings optimized to minimize bleed-through. Image acquisition settings were always kept as constant as possible, and saturation was not allowed. Live cell imaging was conducted in CO_2-independent medium on chambered cover glasses, with controlled temperature at 37 degrees Celsius. 3D image stacks were acquired in most cases. Pixel density (sampling) was always optimized in both X-Y and Z.

Software development. BioImageXD was written in Python [108], with C++ [109] used in some cases. The software utilizes the VTK [58] and ITK [59] libraries and the wxPython [110] library for the graphical user interface. The code is structured and commented and the architecture supports simple addition of new Python modules. The software development was carried out by a multidisciplinary team in a centrally coordinated manner, with an online documentation system, and source code distribution and bug tracking via SourceForge [111]. Regularly performed testing

protocols were devised for testing the most critical functions, and built-in bug reporting systems helped in quality control.

Image processing and analysis. All protocols were defined after extensive testing, and run mostly with the BioImageXD Batch Processor. For 3D fixed cell data, preprocessing often included automatic background subtraction and subset extraction. Integrin clusters were segmented using dynamic threshold and object separation. Cell volumes were defined by smoothing the images with Gaussian smoothing, dilation and erosion, segmenting them with thresholding and finally converting them to polygonal data. For 4D live cell data, image series were first concatenated, then registered with multi-resolution translation registration, and then processed with automatic background subtraction and hybrid median filtering. Segmentation was performed with thresholding and connected component labeling. The BioImageXD motion tracking algorithm was used in some cases, with settings that allowed a lot of variation in the objects being tracked. Traditional colocalization analyses were performed mostly with automatic threshold determination. Advanced colocalization analyses were performed by segmenting one data channel and comparing intensities of the other channel inside and outside the segmented channel. 3D renderings were created mostly with the volume rendering and surface rendering modules of the BioImageXD 3D mode, and videos were created mostly with the BioImageXD animator, using both keyframe animation and camera paths.

Other methods. Flow cytometry experiments were conducted with a Becton Dickinson FACSCalibur flow cytometer, and atomic force microscopy experiments with a JPK Instruments NanoWizard II atomic force microscope, coupled to a Carl Zeiss Axio Observer.Z1-LSM 510 confocal microscope. AFM samples were imaged in contact mode in PBS, with soft cantilevers. Software comparisons were done pairwise, always comparing BioImageXD to some other software on the same computer with the same sample datasets, using carefully defined testing protocols. Simulated images were created with the simulation tool of BioImageXD, mimicking confocal microscopy images from both fixed and living samples. Different levels of simulated noise and integrin clustering were used, with real analysis results as initialization values. Statistical analyses were performed mostly as two-tailed t-tests for unequal sample sizes and variances, with normal distribution checks and approximate Bonferroni corrections.

4. Results

The results obtained in this thesis are described in detail in the original publications I-III. This chapter gives an overview of the most important results. As this thesis consists of both bioimaging method development and biological research, this chapter is accordingly divided into two sections.

4.1. Bioimaging development results

Live cell 4D confocal microscopy protocols were established for the visualization of cell surface receptor molecules. 3D renderings and 4D movies were created, clearly showing for the first time, how the cell surface receptors formed clusters and moved along actin filaments while doing so. By simple but effective image processing (extracting a rectangular section through a 4D cell image and rendering it separately) it was possible to also clearly visualize that the receptor molecules were initially located on the cell membrane, but internalized into the cell when clustering progressed.

For visualizing, processing and analyzing 4D confocal image data, new type of open source software, called BioImageXD [112], was developed (Figure 6). The development was guided by six main criteria, defined according to what was thought that "ideal" bioimaging software should be like:

- *Open*: has open source code and no undisclosed algorithms or background processing.
- *Extensive*: has many features for today's varied and complex applications, including 3D rendering and 4D animation tools, pixel/voxel-based quantitative analyses such as colocalization analysis, several segmentation methods and quantitative analyses based on them, 3D motion tracking, and basic image processing functions such as graphical intensity transfer function adjustment.
- *Usable*: is free, easy to install and use on any computer and operating system, and has a clear graphical user interface.
- *Adjustable*: the parameters of all methods and algorithms are fully adjustable, and it is possible to create command pipelines without programming.
- *Applicable*: is suitable for the current requirements of fluorescence-based multidimensional microscopy techniques, especially high-throughput applications, and contains analysis validation tools.
- *Extendable*: has modular and extendable software architecture, compatible with some existing bioimaging tool libraries, and supporting Open Microscopy Environment (OME).

BioImageXD became one of the largest and most versatile software packages available for bioimage post-processing, and widely used internationally for a very broad array of applications. Compared to other software, BioImageXD was shown to have some rather unique features, such as its operational logic tailored for scientific use, built-in analysis validation tools, and integrated batch-processing support for high-throughput applications. BioImageXD enabled complex and previously nearly impossible image analyses of the clustering, movement, internalization and colocalization of cell surface receptors, in a validated and robust manner from simple confocal microscopy data. Parameters calculated included: cluster intensity, cluster size, number of clusters per cell area, average distance between clusters per cell area, percentage of clusters inside the cell, average cluster distance to cell center per cell volume, colocalization coefficients, and average speed and directional persistence of moving clusters. The software was thus proven to be a powerful and functional bioimage post-processing tool also in practice.

In further application and validation studies BioImageXD was shown to be suitable for quantitatively analyzing small fluorescent particles on and inside cells, using standard confocal microscopy images, and reliably analyzing also cellular internalization from comparatively low-resolution data. BioImageXD was also shown to be able to detect very small differences in for instance colocalization, with statistical significance, and to be able to handle images from different types of sources.

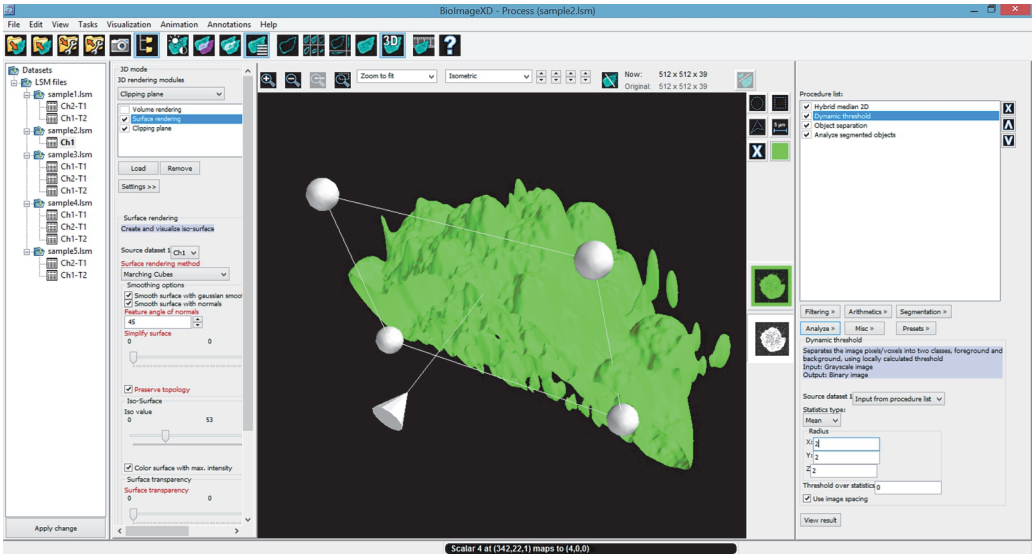

Figure 6. *Screen capture of the graphical user interface of BioImageXD. The user interface is based on a single large window with an intuitive layout. At the top are main tool bars, on the left an internal file tree of loaded data, in the middle a visualization of selected data with the appropriate controls, and on the right settings for the currently active task, showing here a procedure list for segmentation-based quantitative analysis.*

4.2. Biological research results

The collagen receptor integrin α2β1 was shown to reside in raft-type domains on the cell membrane of cells in culture, not colocalizing with caveolin-1. Upon clustering with secondary antibodies, α2β1 integrins started to form larger and larger clusters and move laterally on the cell membrane along cortical actin filaments to caveolin-1 rich areas, eventually internalizing into caveosome-like structures inside the cell. The internalization process required protein kinase Cα, and represents a unique and specific mechanism of integrin entry. Echovirus 1, which uses α2β1 integrin as its receptor, was shown to internalize using a similar mechanism.

When the clustering, internalization and movement phenomena of α2β1 integrin were analyzed quantitatively, it was discovered that they are possibly controlled by different molecular mechanisms: chemical inhibitors that affected clustering the most, such as protein kinase inhibitors Safingol and Staurosporine, had a smaller effect on internalization and movement. In contrast, inhibitors that strongly affected internalization and movement, such as caveolar endocytosis inhibitor Filipin II, did not necessarily have much of an effect on clustering. The clustering and internalization phenomena themselves were found to be rather fast and extensive, and easily measurable under different conditions.

By attaching antibodies against α2β1 integrin onto artificial silica nanoparticles, they were made to behave similarly to Echovirus1 or cluster-inducing secondary antibodies: the nanoparticles specifically attached to cells expressing α2β1 integrin, they initiated cluster formation of the integrins, and they internalized into the cells with the integrins. Quantitative analyses of the intracellular trafficking of the nanoparticles revealed that they entered cells through a macropinocytosis-like process and ended up in caveolin-1 positive structures – just like α2β1 integrin does. These results suggest that it might be possible to use different integrins as address labels to target for instance drug-carrying nanoparticles specifically to different cell types and even different intracellular compartments in the cells. These studies bring the biological results obtained closer to practical applications.

5. Related Published Work

When the work for this thesis was carried out, a lot of related other work was also done in the field of bioimage informatics, resulting in 12 peer-reviewed scientific publications. These publications are not directly part of this thesis, but they illustrate different aspects and applications of the approaches developed, and showcase how they can be widely applied. In all these publications, the author of this thesis was mostly responsible for the bioimaging work specified below for each publiation, in particular confocal imaging, image analysis and work with the BioImageXD software. In addition to the publications listed below, there is a lot of ongoing work not yet published, and some of this is described in the section "6.1.5. Future of BioImageXD".

Kukkonen SP, Airenne KJ, Marjomäki V, Laitinen OH, Lehtolainen P, Kankaanpää P, Mähönen AJ, Räty JK, Nordlund HR, Oker-Blom C, Kulomaa MS, Ylä-Herttuala S. 2003. Baculovirus capsid display: a novel tool for transduction imaging. Mol Ther. 8: 853-862. [113]
This work describes the use of fluorescently labeled baculoviruses as tools to study virus infection with confocal microscopy. The imaging approaches were developed alongside early development of the imaging protocols used for integrin cell surface receptors in this thesis.

Rinne J, Albarran B, Jylhävä J, Ihalainen TO, Kankaanpää P, Hytönen VP, Stayton PS, Kulomaa MS, Vihinen-Ranta M. 2007. Internalization of novel non-viral vector TAT-streptavidin into human cells. BMC Biotechnol. 7: 1. [114]
The internalization of a novel molecular construct was studied using multi-channel confocal microscopy combined with differential interference contrast imaging. There are some basic quantitative measurements of fluorescence intensities and colocalization, but this work is largely based on visualization approaches similar to early visualizations of integrins used in this thesis.

Connors WL, Jokinen J, White DJ, Puranen JS, Kankaanpää P, Upla P, Tulla M, Johnson MS, Heino J. 2007. Two synergistic activation mechanisms of α2β1 integrin-mediated collagen binding. J Biol Chem. 282: 14675-14683. [115]
This study investigates the relationship between the clustering and the different molecular conformations of integrins. Integrin clustering and internalization was induced with the phorbol ester TPA, and visualized with the image processing protocols developed in this thesis work, using early versions of BioImageXD. Simple quantification of cluster internalization by manual inspection of 3D images was also performed, as part of the development process of the automatic internalization quantification tools described in this thesis.

Bergman L, Rosenholm J, Öst A-B, Duchanoy A, Kankaanpää P, Heino J, Lindén M. 2008. On the importance of surface chemical engineering for controlling nanoparticle suspension stability in biotargeting and imaging applications. J Nanomater. Article ID 712514. [116]

This paper is related to early work with the nanoparticles that would later become a major application area of this thesis. Simple visualizations of confocal microscopy data, created with BioImageXD, were used to investigate how well the nanoparticles and their aggregation could be studied with fluorescence microscopic methods.

Karjalainen M, Kakkonen E, Upla P, Paloranta H, Kankaanpää P, Liberali P, Renkema GH, Hyypiä T, Heino J, Marjomäki V. 2008. A Raft-derived, Pak1-regulated entry participates in α2β1 integrin-dependent sorting to caveosomes. Mol Biol Cell. 19: 2857-2869. [78]
The internalization mechanism of α2β1 integrin was investigated in this work, using for instance confocal microscopy. BioImageXD was used to analyze colocalization, and a simple algorithm was implemented in BioImageXD for the quantitative analysis of internalization from fixed images. This algorithm was later used as one means of validating the more sophisticated internalization analysis method developed in this thesis.

Jokinen J, White DJ, Salmela M, Huhtala M, Käpylä J, Sipilä K, Puranen JS, Nissinen L, Kankaanpää P, Marjomäki V, Hyypiä T, Johnson MS, Heino J. 2010. Molecular mechanism of α2β1 integrin interaction with human echovirus 1. EMBO J. 29: 196-208. [117]
The interaction between Echovirus 1 and its receptor, α2β1 integrin, was studied using for instance confocal microscopy. BioImageXD was used to create 3D renderings of integrin clusters, and to properly visualize colocalization and analyze it quantitatively, without resorting to the problematic superimposed red-green images.

Björkbom A, Ohvo-Rekilä H, Kankaanpää P, Nyholm TK, Westerlund B, Slotte JP. 2010. Characterization of membrane properties of inositol phosphorylceramide.Biochim Biophys Acta. 1798: 453-460. [118]
In this work the 4D confocal imaging approaches utilized in this thesis were developed further for a new application area: basic biochemistry research not involving cells. The aggregation of unilamellar model vesicles was visualized with BioImageXD, and sizes of the aggregates were measured using protocols similar to those used in this thesis for integrin cluster size quantification.

Björkbom A, Róg T, Kankaanpää P, Lindroos D, Kaszuba K, Kurita M, Yamaguchi S, Yamamoto T, Jaikishan S, Paavolainen L, Päivärinne J, Nyholm TK, Katsumura S, Vattulainen I, Slotte JP. 2011. N- and O-methylation of sphingomyelin markedly affects its membrane properties and interactions with cholesterol.Biochim Biophys Acta. 1808: 1179-1186. [119]
Here the imaging and analysis of model membrane vesicles, mentioned above, was developed further, in collaboration with biochemists and computer scientists. New tools and methods for segmentation and registration were developed into BioImageXD for quantifying the sizes of different domains on "freely floating" model membrane vesicle surfaces. The goal of this ongoing work is to develop general purpose tools for vesicle research (see also the section "6.1.5. Future of BioImageXD").

Díaz Rodríguez N, Kankaanpää P, Saleemi MM, Lilius J, Porres I. 2011. Programming Biomedical Smart Space Applications with BioImageXD and PythonRules. 4th International Workshop on Semantic Web Applications and Tools for the Life Sciences (SWAT4LS). London, UK. 10–11. [120]
This paper describes how BioImageXD has been studied together with semantic technologies for device interoperability programming approaches. This research, done in collaboration with computer scientists, focuses on new methods of improving interoperability, and BioImageXD was chosen as a use case, because interoperability is of growing importance in bioimaging.

Sukumaran P, Löf C, Kemppainen K, Kankaanpää P, Pulli I, Näsman J, Viitanen T, Törnquist K. 2012. Canonical transient receptor potential channel 2 (TRPC2) as a major regulator of calcium homeostasis in rat thyroid FRTL-5 cells: importance of protein kinase C δ (PKCδ) and stromal interaction molecule 2 (STIM2). J Biol Chem. 287: 44345-44360. [121]
This work describes yet another application area of BioImageXD: isolated confocal optical sections were used to analyze protein translocation to the cell membrane, utilizing multiple segmentation protocols based on the approaches developed in this thesis work. The new protocols enabled fast and very sensitive analyses in situations where differences were not visible by eye.

Paavolainen L, Kankaanpää P, Ruusuvuori P, McNerney G, Karjalainen M, Marjomäki V. 2012. Application independent greedy particle tracking method for 3D fluorescence microscopy image series. 9th IEEE International Symposium on Biomedical Imaging (ISBI). Barcelona, Spain. [122]
This paper, done in close collaboration with the BioImageXD programming lead Lassi Paavolainen, describes in detail the new motion tracking algorithm that was developed into BioImageXD as part of the integrin analyses of this thesis. The tracking algorithm was tested with both simulated and real image data, and compared to other tracking approaches. The algorithm was shown to solve many typical motion tracking problems and to produce accurate results even when used to track challenging objects, such as hundreds of constantly changing integrin clusters (see also the chapter "6.3.3. Motion tracking").

Bergman L, Kankaanpää P, Tiitta S, Duchanoy A, Li L, Heino J, Lindén M. 2013. Intracellular degradation of multilabeled poly(ethylene imine)-mesoporous silica-silica nanoparticles: implications for drug release. Mol Pharm. 10: 1795-1803. [123]
This work is an extension of publication III of this thesis. While publication III describes the cellular attachment, internalization and intracellular trafficking of silica nanoparticles, this paper investigates the intracellular degradation of the nanoparticles. Different components of the nanoparticles were labeled with different fluorescent dyes, and proportional intensities and colocalization of the dyes were quantified with BioImageXD from confocal microscopy images. The sensitive analyses lead to a suggested model of the nanoparticle degradation process and showed that the protocols developed can be used with BioImageXD to study the intracellular fate of multicolor structures.

6. Discussion

6.1. BioImageXD

The most significant result of this thesis is the BioImageXD software. Therefore, the first and largest part of the discussion focuses on the software, covering its design criteria, comparisons to other software, reception by the scientific community, applications and future development.

6.1.1. BioImageXD development criteria

For the development of BioImageXD, six main criteria were defined, according to what was thought that good bioimaging software should be like in general: open (open source code), extensive (has many features), usable (free, easy to install and use), adjustable (command pipelines and full parameter adjustability), applicable (especially for high-throughput applications, with validation), and extendable (having modular and extendable architecture, supporting other libraries and interoperability). The criterion of openness has been increasingly called upon also by other authors [10, 39, 40], with some even appropriately calling it "the most fundamental element" of scientific software [43]. Usability has also been recently pointed out as deserving more attention than what it has been traditionally given [5, 43, 124]. Interestingly, Carpenter, Kamentsky and Eliceiri defined a comprehensive list of bioimage software requirements, coincidentally published in the same issue of Nature Methods where BioImageXD and its six development criteria were published. The list of Carpenter *et al*. includes for instance open source code, extensive features, user-friendliness, validated features suitable also for high throughput applications, extendable modular architecture, and interoperability [5]. The similarity between the criteria of Carpenter *et al*. and the ones defined for BioImageXD already a long time ago is striking, but perhaps not that surprising: even though different researchers would define their "dream software" somewhat differently, there are many common characteristics that most researchers would agree upon, and the criteria defined for BioImageXD development can probably be considered a fairly good general wish-list for modern bioimage post-processing software.

BioImageXD successfully fulfilled most of the six development criteria, as described in publication II. BioImageXD is fully open, with freely available open source code and no undisclosed background processing. It is extensive, having one of the largest collections of tools available in a single bioimaging software package, including important features such as graphical intensity transfer function adjustment, advanced visualization and animation tools, and numerous methods for both voxel-based and segmentation-based quantitative analyses, including motion tracking. All parameters are fully adjustable and combinable into pipelines, and the software is also applicable in many currently important research situations, due to fully integrated 3D/4D

support, data reading only on demand, batch processing, and built-in validation tools. The BioImageXD user interface was designed to be intuitive, and has color coding to assist novice users. However, there is a compromise between adjustability and usability, because in order to be fully adjustable, bioimaging software will inevitably be somewhat complicated to use. BioImageXD has also been designed for proper scientific work, which does not necessarily translate into ease of use. Many users today seem to hope for very simple software solutions, where a few "magical buttons" automatically do what they want, and some commercial software manufacturers are attempting to design their products accordingly. However, this may lead to erroneous results, and for proper scientific work some amount of complexity in software usability should probably be accepted. Another issue that slightly reduces the usability of BioImageXD is that some features are not fully functional in the Mac operating systems. This was caused by inherent problems in the Mac operating systems that the BioImageXD team was unable to overcome, despite numerous attempts. Although the extendibility of BioImageXD is good, owing to the modular design and the support for other libraries and formats, there could be a clearer plugin-type architecture, and more direct interoperability with some other software packages. For high-throughput applications, retaining spatial information of a multi-well plate, for instance, should be supported. Finally, as is the case with all complex and large software packages, but especially open source projects developed with limited resources for testing and bug fixing, there remain several bugs in the software. Bug fixing is constantly being done, but probably some amount of bugs simply needs to be accepted for scientific open source software.

In their list of software requirements, Carpenter *et al*. also point out additional details, such as that the source code should be readily available in a repository such as SourceForge [111], the software should have organized design and development, it should have a format for exchanging data with other software, and it should be compared to other software tools [5]. BioImageXD fulfills also many of these additional details: its source code is available in SourceForge, it has organized design and development, it supports the OME-TIFF format for exchanging data, and it has been extensively compared to other software (publication II).

6.1.2. BioImageXD compared to other software

Publication II describes how BioImageXD was compared to other software tools in terms of features, performance and even practical applicability. Both proprietary and open source software were included in the comparisons: BioImage Suite [52], ImageJ/Fiji [49, 48], Vaa3D [51], VisBio [125], Imaris (Bitplane), Fluoview (Olympus), Volocity (PerkinElmer), ZEN Lite (Carl Zeiss), and MATLAB with the Image Processing Toolbox (MathWorks). The philosophy of proprietary software is so different from open source software, that this alone makes comparisons difficult. Proprietary software does often offer better stability, but adjustability and extendibility are more limited. While the largest ones, like Imaris and Volocity, offer a fairly wide collection of tools, most proprietary software packages have rather limited tool collections. Of the open source alternatives, BioImage Suite has fewer features than BioImageXD,

and is tailored more for medical image visualization and registration. VisBio is designed mostly for visualization only, having for instance no segmentation or batch processing tools, and Vaa3D is tailored for neuronal imaging, having less options in some application areas such as high throughput, tracking and colocalization analysis. ImageJ and its modernized re-incarnation, Fiji, probably come closest to BioImageXD in terms of available features, but the design philosophies of the software packages differ. ImageJ/Fiji is based on numerous plugins developed by a large user community for processing individual images, whereas BioImageXD is a single package based largely on VTK [58] and ITK [59], for processing multidimensional sets of images. While Fiji/ImageJ have more features for working with individual images, BioImageXD is more versatile in areas such as 4D animation, 3D segmentation, batch processing and method validation.

The integrin analyses used as a case study in publication II were attempted also with all the other software listed, but in most cases similar calculations were not possible, as the required features or their adjustability were missing. In those cases where numerical results were obtained, they often differed from the validated results obtained with BioImageXD. While these practical applicability comparisons have many obvious flaws, they do suggest that BioImageXD enables many analyses that are not otherwise easily doable, and that BioImageXD differs significantly from other software also in terms of its practical applicability. All software was also tested for performance speed in file loading, noise filtering and 3D rendering. Overall, BioImageXD was faster than both the open source and proprietary alternatives.

Of course, the software packages used in the comparisons in publication II are only a small subset of what is available. Among other open source projects that have a design philosophy at least somewhat similar to BioImageXD, are for instance Icy, CellProfiler, Endrov, PhenoRipper, cellXpress and pyBioImage; most of them newcomers. Icy [50] is a general-purpose bioimage informatics software package, with integrated support for multidimensionality, VTK [58], and command pipelines. Perhaps the main difference between Icy and BioImageXD is that Icy has a sophisticated graphical tool for setting up command pipelines, but as some authors have also pointed out, Icy and BioImageXD are rather similar [41, 126]. CellProfiler [40, 54], another popular open source alternative, is more clearly different from BioImageXD, intended for high throughput 2D cell segmentation and phenotyping. Some authors have also brought up the differences between CellProfiler and BioImageXD, mentioning BioImageXD to be more suitable for general processing of multi-dimensional images [126, 127]. Endrov [128] is an interesting alternative, capable of both image acquisition and post-processing, and having numerous tools for both visualization and analysis. Compared to BioImageXD, Endrov is targeted specifically for applications where large datasets and extensive annotations are required [128]. The PhenoRipper project [124] has focused on less common but important bioimaging software development aspects: ease of use and fast processing of high content data. Unlike BioImageXD, PhenoRipper is designed for quick, segmentation-free screening of cellular images based on image phenotype similarity, and it is not suitable for quantifying specific features of single cells. cellXpress [126] is

another new and fast software platform for high-content screening, developed for profiling cellular phenotypes. The authors themselves compare cellXpress to BioImageXD, and point out that unlike BioImageXD, cellXpress does not support features such as time-lapse support, 3D rendering and 3D analysis [126]. Although cellXpress has many advanced and innovative features, the software is intended for a single purpose, and is not a general purpose tool in the same way as BioImageXD. Another example of software much more specific than BioImageXD is pyBioImage [55], designed for processing images of germinal centers. An interesting aspect of pyBioImage is that it has been written in Python and C, and the authors also point out that BioImageXD is one of the very few other software packages available with a similar design philosophy. Rather than amending to the Java-based ImageJ, the pyBioImage developers opted to develop their own software using Python, because this enables the use of powerful and increasingly popular visualization and analysis libraries, compatible with Python [55]. Indeed, the fact that BioImageXD makes two popular libraries, VTK [58] and ITK [59], available to the Python [108] community in a single, usable package, is one of the characteristics that separate it from other software. Several articles also point to this as being one of the major noteworthy features of BioImageXD [7, 8, 11, 55, 127].

There are also promising unpublished open source projects under development, such as Microscopy Image Browser by Ilya Belevich *et al*., designed especially for electron microscopy images, but usable also with light microscopy data. Compared to BioImageXD, Microscopy Image Browser has highly advanced segmentation features with useful manual correction tools, but it has limited 3D visualization tools and no high-throughput capabilities. Microscopy Image Browser has not yet been published, but it has been used in a few scientific publications, for instance to segment electron microscopy images of the cochlea [129].

While it can be entertaining to compare bioimage software packages, it is difficult and rather pointless, because they are all very different, and designed for different application areas. The difficulty of software comparisons has been pointed out also by other authors [41, 55]. Different bioimaging software solutions are therefore not direct competitors of each other, but supplement each other, and together offer a wide array of opportunities for researchers. Indeed, it is increasingly being argued, that different software projects should work together, focusing on interoperability [5, 11]. With BioImageXD, interoperability is addressed for instance through support for VTK [58], ITK [59] and OME [27], with support for Bio-Formats [40] being under development. BioImageXD has also several qualities that separate it from many other software packages and make it a good complement to them. For instance, it has one of the largest collections of general-purpose tools, its architecture supports high throughput applications and robust scientific workflows, it has highly advanced visualization and animation tools, it uses VTK [58] and ITK [59] in Python [108], and it contains validation tools. As shown in publications II and III, BioImageXD also enables several complex quantitative analyses that are so sensitive, that they reveal statistically significant results even when no differences can be seen by eye – a quality that is becoming increasingly important in bioimaging [5]. All these characteristics and

the increasing use of BioImageXD in the scientific community indicate that the software does have its place among bioimaging programs and that it can be a significant asset. It should perhaps also be noted, that the most popular software tools are not always the best. For instance ImageJ is in some regards old-fashioned, and does not handle some modern requirements, such as 3D visualization or batch processing, in a particularly user-friendly manner. It has also been pointed out, that the popularity of ImageJ has started to work against itself [24]: The risk of popular plugin software is that the different options become overwhelming for the user [5], and ImageJ has too many plugins, with too much redundancy and variation in quality [24]. Also this means that newer and less popular software packages, such as BioImageXD, have their place.

6.1.3. Reception of BioImageXD

The first beta version of BioImageXD was released in 2006 [130]. Since then, BioImageXD has been reasonably well received by the scientific community. A prime example of this is it being chosen for publication in the special bioimage informatics issue of Nature methods (publication II), where Gene Meyers mentions BioImageXD as one of the major efforts in bioimage informatics, a "good start" [1]. BioImageXD is downloaded from its website hundreds of times every month, and by the end of 2013 BioImageXD had been used or mentioned in at least 75 peer-reviewed scientific publications. Some of these various published applications of BioImageXD are discussed below in the section "6.1.4. BioImageXD applications". By the end of 2013 BioImageXD had also been reviewed in at least 16 scientific publications from bioimage informatics and related fields. Many of these reviews list BioImageXD as one of the standard software packages in bioimage processing [10, 24, 25, 29, 42, 126, 131, 132], while some even classify BioImageXD as one of the most widely used software packages [41]. Some reviews point out that BioImageXD lacks features for certain specific applications [24, 29], but many reviews state the suitability of BioImageXD especially for multidimensional images [39, 41, 127] and command pipeline or high content applications [3, 42, 127]. One review praises the 3D rendering features of BioImageXD as being perhaps the best available [133].

6.1.4. BioImageXD applications

BioImageXD has been designed to be a general purpose tool, not tailored only for some specific applications. Possible applications for the software are therefore nearly endless. Publications II and III describe advanced applications of BioImageXD to visualize and quantitatively analyze the clustering, cellular internalization, colocalization and movement of small fluorescent structures, such as cell surface receptor molecules or artificial nanoparticles. High throughput capabilities are also demonstrated. In addition, the chapter "5. Related published work" contains examples of BioImageXD being used for a variety of advanced 3D/4D visualization tasks of for instance different receptor molecules, different types of nanoparticles and model membrane vesicles [115-118, 121, 123]. Examples are also given of the use of BioImageXD in various types of colocalization analyses [78, 117], and in

segmentation-based quantitative analyses of for instance protein patches on the cell membrane [121], size measurements of vesicle clusters [118], and the degradation of fluorescent structures inside cells [123]. There are also examples of new algorithms developed into BioImageXD, for motion tracking [122] and for the quantification of domain sizes on membrane surfaces [119], as well as a demonstration of BioImageXD being a use case in the development of device interoperability programming using semantic technologies [120]. All these rather complex and multifaceted studies give an overview of the possible applications of BioImageXD, but they are far from being the only reported applications of the software.

Other researchers have also used BioImageXD extensively. Not surprisingly, one of the main application areas is 3D visualization, where BioImageXD has been used for instance in studies of neuronal biology, parasitology, lymphatic disorders, and gene therapy [134-143]. More exotic visualization applications include the use of BioImageXD in visualizing Raman data [144] and in creating advanced visualizations of nuclear microscopy data for cancer research [145]. Studies of colocalization, whether two fluorescent markers occupy the same spatiotemporal location, are very common in bioimaging, but also complex and difficult [17]. BioImageXD has one of the most versatile colocalization analysis tools available, and numerous researchers have used these tools in studies of for instance viruses, mitochondria, retinal cells, nanoparticles, neurons and integrins [79, 138, 139, 146-158]. The third major application area is segmentation and various related quantitative analyses and processing. Here, BioImageXD has been used for instance in the fields of bacteriology, virology and medicine [79, 150, 156, 159-166]. Some applications contain highly advanced segmentation-based approaches [167], and some describe the use of BioImageXD in physics-relates studies [168].

BioImageXD has been developed primarily for fluorescence-based microscopy images, but many of its features are applicable to images coming from any source. Publication III shows an example where BioImageXD was used to visualize atomic force microscopy data. Features such as intensity transfer function adjustment or noise filtering work in the same way for virtually all types of images, and can therefore be easily applied. With some features, such as segmentation, applicability may not be equally straightforward. Fluorescence microscopy results in images with specific staining, high contrast and dark background, unlike images from for instance electron microscopy, phase contrast or differential interference contrast microscopy, all of which are very common. Nonetheless, most algorithms can take any such data as input, and successful results can often be obtained. For instance, in a recent paper BioImageXD was successfully used to analyze cell motility from phase-contrast image series [169], and a lot of recent work by Paavolainen *et al*. has focused on applying BioImageXD for electron microscopy and electron tomography data [170].

BioImageXD is generally designed for single-channel image data, where greyscale (or paletted) image data represents a single set of data, such as one fluorescent marker, an electron micrograph or a picture from a black-and-white camera. The software does support multiple such channels, but multi-component image channels, such as

common RGB color images, have limited support. This is simply because many image processing and analysis algorithms are designed for greyscale data. BioImageXD can open RGB-color images as well, but the use of many features is limited with such data. Nonetheless, BioImageXD has been reported to have been used to for instance determine tumor cellularity from conventional color micrographs of tumor biopsy sections [171]. Another example based on color image data is an unpublished ongoing project where BioImageXD is used to analyze axon growth from nerve biopsies taken after surgery [172].

In addition to scientific journal publications, image analysis enabled by BioImageXD has also resulted in more practical applications, such as a United States patent for a new treatment method for enteritis [173].

6.1.5. Future of BioImageXD

The development of BioImageXD continues actively. In parallel with the current line of development, a new generation of BioImageXD software has been under preparation already for several years [174, 175]. The current software architecture was not designed for software as big as what BioImageXD became, and it is difficult to maintain and improve upon. Therefore, novel software architecture was designed specifically for BioImageXD by Olszewski *et al*., based on stepwise feature introduction, scaled up to fit large software systems [174]. The next generation of BioImageXD is written in Java [176] instead of Python [108], because BioImageXD is so large, that Python was deemed less suitable, and because Java enables easier interoperability with significant Java-based open source projects such as Fiji [49], and easier implementation of the Bio-Formats library [40]. The Java version is still far from releasable, and will be preceded by at least one more release in the current development line, including for instance more tools for electron microscopy data [170]. After that the Python version will be maintained alongside the Java version, but no new features will added.

On the application front, numerous new projects are underway. Three examples are described here, illustrating the use BioImageXD in cell biology, membrane biophysics and biotechnological production.

A) Deen *et al*. are studying hyaluronan synthase, an enzyme responsible for synthesizing hyaluronan directly into the extracellular space. Hyaluronan is a large glycosaminoglycan involved in processes such as cell migration and differentiation, and implicated in pathological conditions such as cancer [177]. Deen *et al*. have previously used BioImageXD for colocalization analysis [178]. In a new project, BioImageXD motion tracking is applied to track the trafficking of hyaluronan synthase to and from the cell membrane (Figure 7 A), a phenomenon essential in hyaluronan synthesis [178]. This project extends the applications of the BioImageXD motion tracking algorithm to a new area: the tracking of thousands of endocytic vesicles.

B) In collaboration with Björkbom *et al*. BioImageXD has previously been used to quantify lipid domains on giant unilamellar vesicles [119]. This vesicle quantification work was complicated and laborious, and now new tools are being developed into BioImageXD for simple and robust analysis of model membrane vesicles (Figure 7 B). These tools are utilizing new types of analysis methods and simulations, the goal of the project being to develop repeatable workflows that enable, for the first time, researchers to easily image and quantify vesicle domains.

C) In a new collaboration with Dandapani *et al*. BioImageXD is being applied to somewhat different type of microscopy data, confocal images of *E. coli* bacteria (Figure 7 C). Dandapani *et al*. study the repair mechanism of *E. coli* iron-sulfur clusters that have the ability to exchange electrons for the catalytic activity of the enzymes that contain them. Iron-sulfur clusters are sensitive to oxidative stress, and understanding their repair mechanisms can help improve the biotechnological production efficiency of commodities such as biofuels. [179] In this project, living *E. coli* bacteria, containing a fluorescent reporter for iron-sulfur cluster damage, are imaged with a confocal microscope to confirm novel speculations that in addition to oxidative stress, also cold stress could damage iron-sulfur clusters. Images of the bacteria are segmented and analyzed for fluorescence intensities with BioImageXD.

Dandapani *et al*. have not utilized imaging before, but relied on plate-reader-type approaches to measure fluorescence. The imaging project now commenced illustrates an important point: not only can the use of quantitative bioimaging reliably confirm the results from other techniques, such as a plate reader, but it can also reveal completely new information. In this case, advanced image analysis showed a significant correlation between two fluorescent markers, which cannot be seen with a plate reader, and which indicates that cold stress can indeed induce iron-sulfur cluster damage similarly to oxidative stress. This study exemplifies a general new trend in biological research: conventional flow cytometry and plate reader -type assays are giving way to high content cellular imaging [3]. This trend is also reflected in the emergence of new types of devices, such as flow cytometers capable of imaging every cell they measure [180]. The realm of bioimaging continues to expand, and therefore also the need for versatile software such as BioImageXD.

BioImageXD is a relatively widely used tool, playing a role in the European imaging community, where the project also participates in a number of international activities. Such activities can be expected to become more significant in the future, and lead to better collaboration between different software projects. However, BioImageXD faces many of the same serious challenges as other open source projects (see the section "6.2. Open source software development" below), most notably lack of proper resources for future development. It seems difficult to get academic resources even locally, despite the project being highly successful. This is one reason why the BioImageXD team is also considering alternative development models for the future, most notably a centralized or commercialized service model (discussed further in the chapter "7. Conclusion and the future of bioimage informatics").

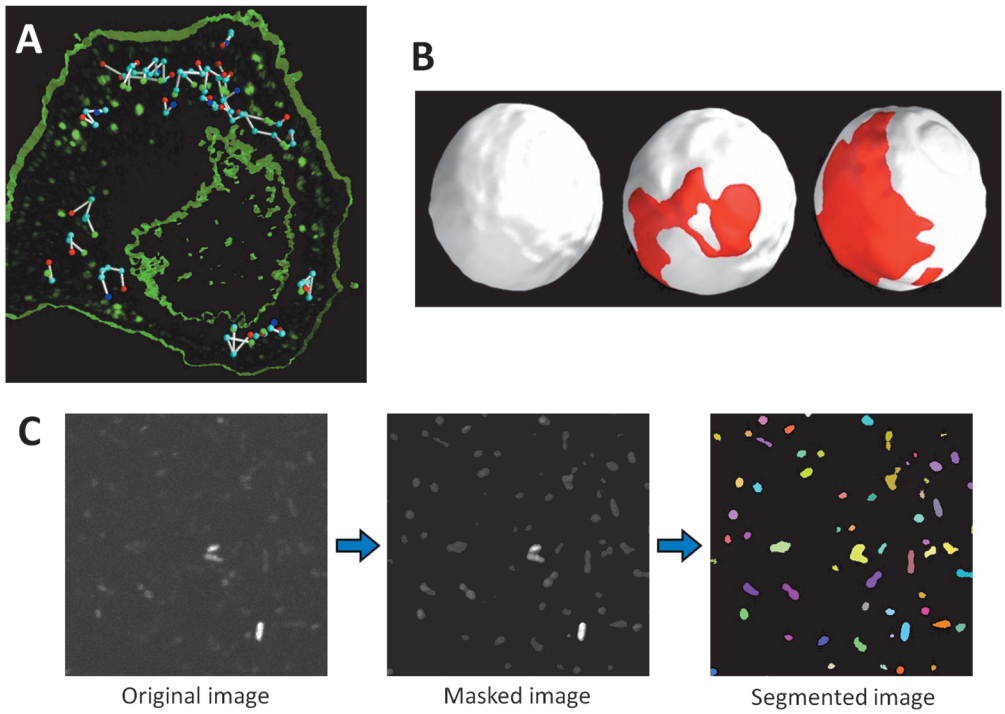

Figure 7. *New applications of BioImageXD for confocal microscopy data.* ***A.*** *Motion tracking thousands of endocytic vesicles. A few sample tracks are shown, rendered in 3D with a 3D surface rendering of a section of a cell.* ***B.*** *Analyzing domains on model membrane vesicles. Sample 3D renderings of vesicles are shown, with varying amounts of saturated/unsaturated lipid domains (red/white). The domain sizes are quantitatively analyzed with new tools being developed into BioImageXD.* ***C.*** *Quantitative imaging of* E. coli *iron-sulfur cluster repair mechanism. A sensitive protocol was developed for masking the bacteria from the background despite largely varying intensities, after which segmentation-based analyses can be conducted. Images are single optical sections.*

6.2. Open source software development

A computer program is "open source" when its source code is available to anyone publicly, published with a license that allows its free use and modification. Open source projects have become increasingly popular in recent years, and the internet is largely based on open source software. Well known and popular examples include the Linux operating system, the Python programming language and the Open Office suite. [43] In scientific imaging it is important to know exactly what is being done to the images when they are being processed, and to be able to exactly control and even modify the parameters and algorithms as needed. It is increasingly being pointed out in the scientific community, that scientific software should be open source [2, 5, 7, 11, 39, 40]. In a recent article, Cardona and Tomancak argue that commercial software companies nearly always hide their algorithms and other details in the name of

generating revenue, and that this is not acceptable for scientific work [43]. Rueden and Eliceiri agree, saying that while proprietary software products can be powerful, they are not sufficiently transparent [10], and Li *et al*. mention open source products as being even more powerful than proprietary ones [41]. Notably, Carpenter *et al*. point out that while it is often thought that the open source model is not compatible with commercial interests, in reality this is not the case, and open source software has often been shown to be beneficial also from a commercial point of view [5].

The need and usefulness of open source software in scientific work can hardly be argued, but the open source software model does have several problems. Typically, open source projects have fewer resources than commercial ones, and there is less organized development and bug fixing. Hence the quality of open source projects varies greatly, without being controlled, and the programs often have bugs even in critical features. Continuity is also a challenge, as especially smaller projects often rely on only one developer [43]. Furthermore, in bioimage informatics, open source software development requires seamless interdisciplinary collaboration [43], which is not always easy to arrange. There are already countless open source software packages and plugins, and more are constantly becoming available [131]. The complexity and sheer number of programs require common ground rules to be established and interfaces to be built, so that the different software projects can more easily communicate with each other, share information and be used together [5, 11]. There are some attempts to organize this, such as the Open Bio Image Alliance in Europe [181] and some NIH-funded projects in the United States [5]. However, as different software projects use different internal architectures and programming languages, making them work together is not simple. It would probably be appropriate to consider also other options, such as centralized image analysis services, as discussed in the chapter "7. Conclusion and the future of bioimage informatics".

6.2.1. Main challenge: software development not properly recognized

An enormous problem with open source projects in life sciences is that software development is often not considered a "scientific activity". Software developers are not respected, they get only middle author positions in scientific publications, and funding agencies are reluctant to support software development, and they often strangely favor purchasing expensive proprietary licenses rather than supporting open source development [5, 43]. These problems certainly became very apparent during the development of BioImageXD, and slowed down the progress of the project considerably. Several authors now very appropriately argue, that this needs to change: software engineering is often required in biological research and produces tools that have a wide impact, and it is high time that this be properly acknowledged by both researchers and funding agencies [5, 43]. It has even been suggested that software downloads should be considered comparable to scientific journal publications [43] – a much welcomed suggestion.

It could be argued that scientific work has no value whatsoever without the necessary transparency, and therefore the open source software model is the only viable

possibility for scientific work. Whatever challenges the open source model may have, the scientific community needs to tackle them, because in most cases there is no other option.

6.3. Aspects of bioimage informatics

Analyzing bioimages accurately and reliably is understandably not a simple task. This chapter discusses some aspects of bioimage informatics that are important, but sometimes overlooked.

6.3.1. Simulations

In this thesis, simulated integrin clusters with sample cell membrane polydata were used to confirm the accuracy of the clustering, internalization and motion tracking analyses conducted (publication II). Since even a single cell easily contains hundreds or thousands of integrin clusters undergoing complex 4D dynamics, checking analysis results manually is not possible even for a single cell. Therefore in this case, as in general with applications involving large amounts or complex data, simulations or other data with a known "ground truth" are the only realistic option for method validation [39, 131, 182].

There are some ongoing attempts at assembling databases of test material for validation purposes [40] or even creating software specifically for rendering realistic microscopy images, such as SimuCell [183]. However, considering that many authors have written about the obvious importance of method validation [5, 39, 131, 182, 183] or even done it themselves with simulations [33, 183, 184], it is surprising that so few general purpose bioimaging software packages contain validation tools. This is one reason why such tools were included in BioImageXD. The simulation tools in BioImageXD are adjustable and offer a good start, but creating realistic simulations is understandably difficult [131, 183]. For example, while the simulations used in this thesis did contain varying degrees of simulated noise, they were not passed through a point spread function (PSF), which would have made them more realistically resemble images from a confocal microscope. However, the BioImageXD simulation tools are constantly being improved, and for instance PSF filtering has already been added.

It would be important for the bioimage informatics community to pay more attention to validation. Simulations help not only in validating methods, but also in making comparisons between different methods [5, 182]. Ideally, simulations should be complemented by actual test data with a known ground truth, but unlike with simulations, obtaining the ground truth for actual data can be very difficult [131].

6.3.2. Resolution

The low resolution of light microscopic techniques is a continuous challenge in modern bioimage informatics. It is easy to forget that for instance a positive colocalization result does not necessarily mean that two proteins interact, it just means that they occur within the same spot, and the size of the spot is determined by the resolution of the microscope, and it is rather large compared to the size of the proteins. In this thesis, the low resolution was particularly challenging for the internalization analyses, which are further hampered by the fact that the axial resolution is important for them, but much lower than the lateral resolution. How can one be sure, if a molecule on the cell membrane actually goes inside the cell, when the axial resolution of the imaging device is much lower than the thickness of the membrane? To confirm that the internalization analysis actually worked, it was checked in numerous ways, such as by calculating object distance to cell center, by comparing to differential staining analysis, by comparing to colocalization results against internalization markers, and by using simulated test datasets (publication II and a lot of unpublished work). All checks consistently yielded expected results, even to the extent that it was surprising. Conducting such checks would in general be important every time, when the low resolution of the imaging device could be expected to affect results. The results should also be treated with caution every time, and other methods should be used to confirm them further.

In this thesis, the internalization analyses were validated further in publication III by using flow cytometry and to some extent the high resolution capabilities of atomic force microscopy (Figure 8). Indeed, as discussed earlier, there are also many light microscopic super-resolution techniques now available, which could help in dealing with the resolution challenge, in addition to AFM. However, compared to standard confocal imaging, super-resolution methods are still often limited in robustness and applicability, and often slow [38]. While it would be good to see AFM used more, especially since it can be used simultaneously with optical imaging, AFM also has its limitations. It could therefore be argued that it can be highly beneficial to develop tools such as the internalization analysis of this thesis, which operate on standard low-resolution confocal data, but are still extensively validated. Super-resolution techniques are not a "magical" solution to the resolution challenge, at least not yet.

How, then, is the internalization analysis of BioImageXD able to produce reliable results despite the low resolution? One part of the explanation could be that although a fluorescent spot is blurred by the PSF, its precise location can often still be calculated, as indicated by some single molecule localization studies [131]. The BioImageXD internalization analysis can utilize both the center of mass of an object and all of its voxels separately, which may improve accuracy. In this case the internalizing objects being studied also tend to go "deep" into the cell, and not just slightly under the membrane surface, which helps in the detection of their internalization.

However, perhaps the most important explanation is that when sufficiently many cells are analyzed, inaccuracies caused by the low resolution no longer matter as much, and even small differences can become statistically significant. This translates into a crucial "take home message" in quantitative bioimaging: Absolute values are not needed in most cases. Relative differences, such as those between a sample and a control, are sufficient. If there is a statistically significant difference between these relative values, then that is enough, and various inaccuracies caused by the low resolution or other factors, can be tolerated to a large extent. The many super-resolution techniques now available can be tempting, but researchers should not think that going for the highest resolution is automatically the best option. Instead, using a robust and well applicable method that can still give good results, such as the internalization analysis of this thesis, can be a better compromise. One should also refrain from extra post-processing steps done only to increase resolution, such as deconvolution, unless they are absolutely necessary. Such post-processing tends to introduce additional error sources to techniques that already have too many error sources to control [185].

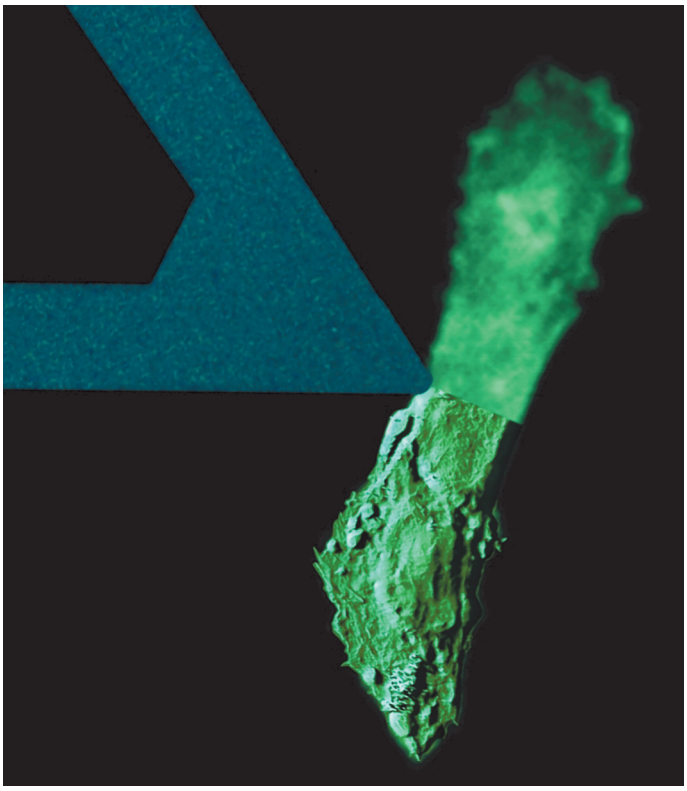

Figure 8. *Composite image illustrating simultaneous use of confocal and atomic force microscopy. The top part of the cell is a confocal microscope image (maximum intensity projection), and the bottom part an atomic force microscope image. The difference in resolving power can be seen. The image of the tip of the atomic force microscope has been enhanced to be visible in the confocal image.*

6.3.3. Motion tracking

Motion tracking means that for each segmented object in a time point, the corresponding object is identified in the following timepoint, and in this way the motion of the objects can be tracked and quantitatively analyzed [18]. Motion tracking has been done since the early days of fluorescence microscopy [18]. Today, motion tracking is in increasing demand [7] and popular even in high-throughput applications [9, 184], but tracking is very challenging, because it consists of many steps. The first step is segmentation, which in itself can be demanding from any type of data [40], but especially from time lapse data, where the image quality is often lower, and the characteristics of the objects being segmented can change over time for instance due to clustering [28]. Tracking often requires also additional processing, such as registration to compensate for unwanted movement [18], and registration with cellular images is another step that already in itself can be quite demanding [40]. Finally, the actual tracking procedure is not very simple, as the tracking algorithm often needs to be able to deal with phenomena such as appearing and disappearing or merging and splitting objects, and low temporal resolution [28].

The BioImageXD tracking algorithm was developed specifically to be able to deal with many of the problems of motion tracking, such as appearing, disappearing, merging and splitting objects [122], and it has been successfully used for instance to track integrin clusters, which frequently change in shape and dynamics (publication II). The tracking algorithm of BioImageXD is a general-purpose tracking solution, capable of tracking equally well a few cells or hundreds of small objects within a cell [122, 170]. However, the algorithm is rather simple in design, not supporting for instance state-of-the-art machine vision approaches. One goal with the development of the algorithm was just to make a functional multi-purpose tracking algorithm available to the users of BioImageXD, and whether it was state-of-the-art or not was of secondary importance. Despite its simplicity, the BioImageXD tracking algorithm has performed even surprisingly well with both simulated and real data, and compared favorably even to the highly regarded Jaqaman method [122]. Importantly, motion tracking should be performed on 3D rather than 2D data [28], and just like everything else in BioImageXD, the tracking algorithm natively operates on 3D data.

A significant problem with biological motion tracking is that a single object often undergoes several different types of movement. On all scales in biology, from migrating birds to proteins moving inside cells, the movement trajectories are complicated, consisting of components such as directed movement, random diffusion and curvilinear motion. Furthermore, the dominating type of movement may vary over time, objects may temporarily get "trapped" inside confined spaces, which changes their movement characteristics, and the type of movement is often not known beforehand. [28, 33, 131] Many researchers overlook this aspect of motion tracking, and traditionally tracking algorithms consider only one type of movement [33]. This is true also in the case of the tracking algorithm of BioImageXD, and terms such as "speed" should in fact not be used for the trajectories of the integrin clusters, for example, as this implies typical directed movement, such as that of a car. The

movement of the integrins is likely to be much more complex. Indeed, unpublished studies indicate that there are at least two components of movement, simultaneously affecting the integrin clusters: a slower, more directional movement, possibly taking place along actin filaments as shown in publication I, and a faster, more random movement, where the clusters move here and there (Figure 9). The directed movement is typically more visible when the interval between imaging timepoints is longer (minutes), whereas the more random component becomes apparent with shorter intervals (seconds). Interestingly, Huet *et al*. speculate that typical movement along actin filaments might be like Brownian motion, whereas movement along microtubules would be more directional [33]. This does not seem to fit the observations made with the integrin clusters, but any evaluation of the different components of movement of the integrin clusters are only preliminary at this stage, and the dominating component may also vary over time and between individual cells. In any case, it can be said with a fair amount of certainty, that the integrin clusters do exhibit complex patterns of movement, and the tracking analyses conducted in publication II are not sufficient to account for them. Nevertheless, even though the BioImageXD tracking algorithm can only analyze one type of movement at a time, its flexibility would in principle allow it to be used to identify the different movement components by running it repeatedly for the same data, with different settings. However, the time interval used during image acquisition would also affect the detection of the different components.

The BioImageXD tracking algorithm has already been used for many different applications, such as tracking integrin clusters (publication II), tracking thousands of small vesicles in a cell (section "6.1.5. Future of BioImageXD"), tracking cells from phase-contrast images [169] and tracking cancer cells moving within a 3D matrix (unpublished data). Interestingly, many tracking algorithms have their basis in military radar applications [131], and the overall principles of cell biological tracking are largely the same as for instance those recently described for the tracking of swarming malaria mosquitoes [186]. Thus, the BioImageXD tracking algorithm can in principle be used to track almost anything, provided that the user has sufficient, and rather extensive, expertise. Nonetheless, the BioImageXD tracking feature needs to be refined to better take into consideration the different types of movement, and researchers should always be cautious when interpreting any cell biological tracking results.

6.3.4. Visualization vs. analysis

When this thesis work was started, making clear 3D renderings and videos of 4D confocal microscopy images was in itself something new, and sufficient for a publication. Today, the situation is quite different. Advanced 4D imaging techniques have become commonplace, images and videos themselves are no longer enough for scientific publications, and quantitative analyses are always needed. This development has been fast, but it is also useful and necessary. Images contain enormous amounts of information just waiting to be extracted, when one has the right tools and the right know-how. This is precisely why BioImageXD was also developed to contain numerous tools for quantitative analyses (Figure 10).

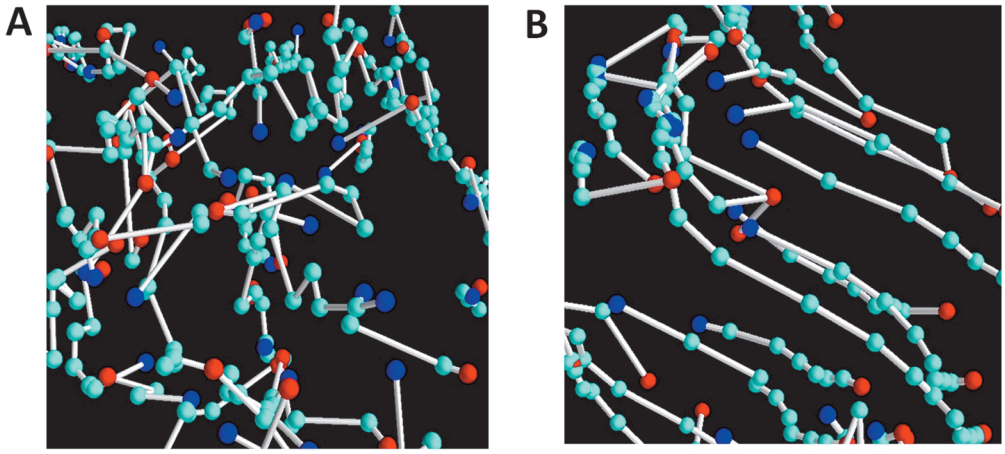

Figure 9. *Two components of movement of α2β1 integrins.* ***A.*** *More random movement, which also often seems faster.* ***B.*** *More directed movement, perhaps indicating transport along actin filaments. Both images are 3D renderings of motion tracks from parts of a cell. Dark blue balls represent track starting points, and red balls track ending points. Imaging time interval 3 min.*

Many authors stress the importance of quantitative analyses [2, 18, 39, 40, 131, 187], referring to reasons such as that the often unclear nature of biological data requires statistical analyses for identifying differences, visual inspection is not feasible with large data amounts, and quantifications are needed for applying computational models to the observations. Publications II and III of this thesis present examples of studies where quantitative analyses were absolutely necessary, and without them almost no valuable data would have been obtained. However, sometimes one may get the impression, for instance when visiting bioimaging conferences, that the transition from qualitative to quantitative microscopy has been extremely fast, and all that researchers now care about are numbers. On the other hand, the same conference one year later may give the opposite impression: researchers do want visualization tools also. Indeed, amidst the rapid development of quantitative bioimaging, it might be good to remember that visualization tools are still also very much needed. Humans are to a large extent a visual species [13], and the famous saying, "a picture is worth a thousand words", illustrates this well [3]. Several authors agree that while quantitative analyses are necessary, also visualization is needed in most applications, for instance as a first step to inspect the acquired data before analysis, or to check the analysis result afterwards [2, 7, 40, 131]. Visualization tools are often useful also while setting up the parameters of a quantitative analysis, and in most cases BioImageXD shows also a visualization of the data being processed, even if the process underway is intended for producing numerical results.

With BioImageXD the goal has been to offer a balanced set of features for both visualization and analysis, and as indicated by the typical applications BioImageXD is

used for (section "6.1.4. BioImageXD applications"), both the analysis and visualization tools are heavily employed. Perhaps it would be safe to say that there cannot be one without the other.

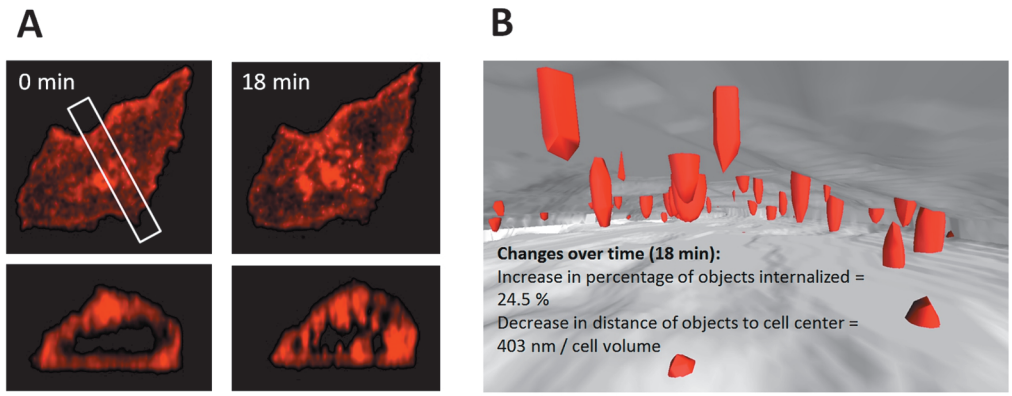

Figure 10. *Change in confocal microscopy imaging of integrin internalization in the last decade.* ***A.*** *Ten years ago cross-sections of cells were rendered and internalization evaluated only visually, as in publication I and in this example* [115]. ***B.*** *Now sophisticated renderings from inside whole cells can be created, as in this example, and when both the integrins and the cell membrane are segmented, as in publication II, internalization can be quantitatively analyzed with parameters such as percent of internalized object voxels and normalized average distance of objects to cell center of mass.*

6.4. Biological significance of this work

6.4.1. Visualizations of α2β1 integrin

This work enabled the visualization of α2β1 integrin clustering, internalization and movement along actin filaments very clearly (publication I), resulting in many further studies aimed at clarifying the mechanisms of how α2β1 integrin, and collagen receptor integrins in general, operate. The visualizations also significantly contributed to the realization that α2β1 integrin function is unique among the integrins, as α2β1 integrin internalizes via a previously unknown caveolin-1-related mechanism (publication I). Many of the findings, such as the clustering and actin-related movement, have received support in subsequent studies [78, 117]. Integrins are important in numerous physiological and pathological processes, and particularly because α2β1 integrin is unique among integrins, the biological results of this thesis may have implications for some medical conditions and their treatments, such as blood clotting and cancer metastasis. α2β1 integrin is also the receptor for human Echovirus 1, making these results useful in the field of virology.

Some of the biological results of publication I indicate that α2β1 integrin normally resides in lipid rafts together with GPI-anchored proteins, and that the internalization

of the integrin takes place via caveolae. However, the existence and exact nature of lipid rafts has recently been debated, and live cell studies with super-resolution STED microscopy have suggested that lipid rafts may actually be very small and transient, approximately 20 nm in diameter and lasting only tens of milliseconds [188], contrary to the interpretations presented in publication I. Also the internalization mechanism of α2β1 integrin has been clarified in further research, and the internalization is nowadays believed to take place not exactly via caveolae, but via a macropinocytosis-like mechanism, which eventually leads the integrins into caveolin-1-positive intracellular structures [78]. These newer results do not mean that the biological findings of publication I would be "wrong", rather, they just illustrate the scientific process: publication I was completed ten years ago, and it is only natural that some of the discoveries have been refined since then. It should also be noted that despite the newer results, the most basic findings of publication I still hold today: α2β1 integrin forms clusters, moves laterally, and internalizes in a unique manner that involves caveolin-1.

6.4.2. Quantitative analyses of α2β1 integrin

This thesis work enabled highly sensitive and accurate quantitative analyses of α2β1 integrin clustering, internalization, movement and colocalization with caveolin-1, and these analyses were shown to be possible also in a high-throughput manner. While the biological results of these analyses mostly confirmed earlier observations (see above), some new information was also obtained, most importantly the notion that the clustering and the internalization are likely controlled by different molecular mechanisms (publication II). The lateral movement of the integrins seems to be linked to the internalization, but not so much to the clustering. While these results are only tentative, also other recent publications have indicated similar results [78, 117]. An interesting contradiction is that the results from publication II might suggest a different role for protein kinases than publication I, but these results are inconclusive, and more experiments would be needed. It is also possible that these contradictions are simply another indication of the fact that different and still partly unknown mechanisms regulate different aspects of the function of α2β1 integrin, and that different ligands, such as antibodies, Echovirus 1 or collagen, are likely to trigger different operational mechanisms of the integrin [117].

Further quantitative image analysis studies revealed that silica nanoparticles coupled to antibodies against α2β1 integrin could induce the clustering and specific internalization process of the nanoparticles (publication III). During internalization, the nanoparticles colocalized with the macropinocytosis marker GM1, Caveolin-1 and the late endosome marker Rab7, but not with other internalization markers (publication III). This indicates that the particles are internalized via a specific route. Notably, the implied route matches not only with the Caveolin-1-dependent internalization model of α2β1 integrin presented in publication I, but also with the refined internalization model that includes a macropinocytosis-like mechanism [78].

The nanoparticle studies also illustrate that it is possible to "mimic" a naturally occurring internalizing small particle with artificial particles. Echovirus 1 can bind to non-active α2β1 integrins and induce clustering and the signaling necessary for internalization (such as protein kinase Cα), without changing the conformation of the integrin to the active state [117]. As this thesis shows, an artificial nanoparticle can behave similarly, and trigger specific internalization mechanisms, even if the address label on the particle is not similar to an "activating" natural ligand such as collagen.

Rajendran *et al*. have recently brought attention to the fact that current drug delivery strategies focus on targeting drugs to certain cell types, but do not address intracellular targeting, although the final targets of many drugs are specific intracellular compartments [189]. In this thesis also specific intracellular targeting of potential drug-carrying nanoparticles is demonstrated. The findings therefore have significant potential in the future development of specific therapeutic applications. The nanoparticle internalization mechanism now discovered also has added value, because it is unique among the integrins. Different integrins are expressed on different cell types and internalize via different mechanisms, and accordingly, nanoparticles have been successfully targeted to different cell types using different integrins as address labels. The results of this thesis imply that different integrins could even be used to target nanoparticles to different intracellular compartments. α2β1 integrin is interesting also because it is not recycled back to the cell membrane in the same way as other collagen receptor integrins, but instead seems to enter a non-recycling degradative pathway [79]. This might give nanoparticles guided to cells via α2β1 integrin more time to release their payloads intracellularly. On the other hand, if the target is degradation, also the payload might be degraded. Further studies with the nanoparticles used in this thesis indicate that the particles are indeed degrading inside the cells, but the process is slow and should allow enough time for a payload to be effective [123]. Knowledge of the internalization mechanism and intracellular fate of the nanoparticles not only helps in drug delivery, but it may also be valuable when designing the particles to be suitable for the pH and other environmental conditions of the compartments and organelles they will be exposed to [189].

6.4.3. Other aspects of biological significance

Interestingly, technical method development for BioImageXD has sometimes resulted also in biological information that has been found useful by the biomedical research community. For example, a report introducing the batch processing features of BioImageXD [190] contained as examples some tentative analysis results of T-plastin (an isoform of the actin-bundling protein fimbrin) colocalizing with α2β1 integrin. These analysis results were later cited by Le Goff *et al*. as supporting their new findings of L-plastin (another fimbrin isoform) and β1 integrin forming complexes in adherent cells [191].

Overall, however, the significance of this thesis lies not so much in the biological findings obtained, but in the imaging tools and methods developed. Importantly,

these tools and methods are widely applicable for almost any fluorescent molecule. A major part of the biological significance of this work is therefore in all the potential future discoveries that are made possible especially by BioImageXD, in a variety of fields. Numerous interesting biological results obtained with BioImageXD have already been published (section "6.1.4. BioImageXD applications"), and many more are likely to follow. The potential for biological discovery, enabled by this work, is substantial.

7. Conclusion and the Future of Bioimage Informatics

The aims of this thesis formed a natural continuum, from developing 4D imaging protocols, to developing software for properly visualizing and analyzing the 4D imaging data, to finally applying the techniques developed to something new and more practical. All the aims were successfully reached: clear 4D visualizations of α2β1 integrin clustering, movement and internalization were obtained for the first time, one of the largest and most versatile open source bioimaging software packages, BioImageXD, was developed and released, and imaging and analysis protocols were applied to study and develop prototype drug carrying nanoparticles. The aim to develop software took a very long time to complete, but this is understandable, considering that BioImageXD is very large and complex, it was developed in a new field of science with limited resources, and the project faced many of the severe difficulties that open source software development in life sciences often faces. A major underlying aim of the thesis was to promote the use and development of advanced bioimaging. This aim was also successfully reached, especially through the BioImageXD software, which was well received by the scientific community and has been used and referenced in numerous scientific publications. BioImageXD was also able to bring to the scientific community many unique features not previously easily available, and enables many bioimage post-processing tasks that previously would have been difficult to carry out. All in all, this thesis has made a significant and hopefully long-lasting contribution to a new field of science, bioimage informatics.

Unfortunately, the future of BioImageXD, and bioimage informatics in general, is not straightforward, despite initial success. Many authors have pointed out considerable challenges of bioimage informatics, including the following:

- Bioimaging requires multidisciplinary skills in for instance biology, imaging, engineering and software production [11, 24].
- Techniques such as optical sectioning microscopy and live cell imaging are not easy, and require considerable expertise [15, 24, 185].
- Many image processing tasks are complex and difficult, also tasks that may seem simple, like colocalization analysis, and even choosing which algorithms or methods to use can be difficult for non-experts [17, 40, 182].
- It is very easy to manipulate bioimages during post-processing, also incorrectly or inadvertently, if one does not know exactly what to do and how [29, 45].
- As image quantification becomes more and more common, the risk of false confidence in the results increases [2].
- Bioimaging involves an array of skills that are difficult for the average researcher to master [5].

- Tailor-made software and programming skills are often required in bioimaging [25, 42].
- A recent survey showed that biologists estimate their own image analysis skills to be only average, and that expertise in biology and expertise in programming do not correlate [43].
- Biologists would require easy-to-use bioimaging software solutions, but such solutions are difficult to produce [2, 5].
- Post-processing bioimages is very slow, and often requires substantially more time than what researchers predict [24, 124].
- The requirements for creating successful bioimaging tools are very different from the requirements for conducting successful conventional biological research, and there are profound difficulties in getting the scientific community to recognize bioimage informatics as a proper scientific discipline [2].

Finding ways to tackle these challenges is not easy. One possible approach might be to centralize bioimage post-processing software services to financially independent units, which might be either independent commercial undertakings or special units of universities or other research institutions. Image acquisition is already often carried out in core facilities, but separate units would be needed on the software side. These units would not sell software, but rather services in bioimage analysis and processing, ranging from simple consultation to finalized numerical results and even tailored algorithm development. Such a service model would solve many of the problems listed above. Bioimages would be handled by experts, results would be reliable and obtained substantially faster, and researchers would be freed to focus on the actual biomedical research. The question is, will researchers understand this and be willing to "trust experts" rather than wanting to do everything themselves, and will they be willing to pay for this, in order to make the model sustainable?

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Turku, June 2014

Pasi Kankaanpää

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