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Analysis of live cell images: Methods, tools and opportunities

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ABSTRACT

Advances in optical microscopy, biosensors and cell culturing technologies have transformed live cell imaging. Thanks to these advances live cell imaging plays an increasingly important role in basic biology research as well as at all stages of drug development. Image analysis methods are needed to extract quantitative information from these vast and complex data sets. The aim of this review is to provide an overview of available image analysis methods for live cell imaging, in particular required preprocessing image segmentation, cell tracking and data visualisation methods. The potential opportunities recent advances in machine learning, especially deep learning, and computer vision provide are being discussed. This review includes overview of the different available software packages and toolkits.

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1. Introduction

Starting with Antonie van Leeuwenhoek, who published his discovery of bacteria, blood cells and muscle fibres in a number of letters to London's Royal Society in the late 17th century, optical microscopy has become an indispensable tool for scientific discovery. Imaging cells and tissues using methods such as automated high-content and high-throughput microscopy have offered new insights into biology. This review clarifies the role imaging can play in gaining insights into biology and describes the extraction of quantitative information that will aid in this task. Quantitative imaging will be required at all anatomical scales ranging from the sub-cellular to the organ level, where structure, form, organization, and most importantly function, are essential for a complete characterization of the organism.

In this review we will focus on the sub-cellular and the cellular scales of living organisms. The characteristic structure and function at each scale is associated with cues manifest as biomolecules which are made to express near visible or visible electromagnetic energy endogenously, or are tagged appropriately to respond to optical stimuli. Optical instrumentation will then convert the expressed energy into a digital signal that will be analysed to glean structure and function for the specimen. Imaging reveals both structure and function of a single cell or a collection of cells.

1.1. Imaging the cell

The nucleus was the first cellular organelle to be discovered by a microscope. Leeuwenhoek observed a "lumen", the nucleus, in the red blood cells of salmon. Today, tagging or binding specific molecules is now the preferred way to delineate the nucleus. Although, histology has leveraged the use of hematoxylin and eosin stains to great advantage for capturing tissue architecture, that specific labelling technology is of limited value for live-cell imaging. Fluorescence microscopy is resorted to localize the binding location in the chromosome of a fluorescent probe; excitation through the leverage of fluorescence phenomena, in turn, delineates the nucleus. Selected examples are shown in Fig. 1. Thus, DAPI (4',6-diamidino-2-phenylindole) is a popular fluorescent stain that binds strongly to A-T nucelotide rich regions in the DNA. A variegated and sampled landscape can be obtained by using fluorescent in situ hybridzation (FISH) probes which localize specific DNA and RNA level expression in the cell. The cytoskeleton composed of the water-rich cytoplasm and various filaments is imaged through the use of fluorescence. Phalloidin is often used by labelling it with fluorescent analogs and then subsequently staining actin filaments. Further emblematic of the adoption of this approach has been the discovery of the green fluorescent protein [1] and the wide-spread use of microscopy methods such as confocal and two-photon optics that have dramatically transformed live cell imaging. Given the leverage that the optical spectrum offers in capturing multiple structures and functionalities (cellular mechanisms and signalling), the colour revolution has spurred the wide-spread use of imaging both for high-content and high-throughput [2].

The desire to understand intercellular and intracellular processes has driven the development of super-resolved fluorescent microscopy [3,4]. To monitor organ formation or indeed the development of an entire organism in 4D (3D + t), the concept of *in toto* imaging [5,6] has been developed. With the help of advanced biosensors [7] it is now possible to report the activation states (e.g. conformation and phosphorylation) of endogenous proteins with minimal perturbation. In 2005 three different groups established Channelrhodopsin-2 (ChR2) as a tool for genetically targeted optical remote control [8–10]. Rapidly developing optogenetics techniques [11] now allow the fast and specific excitation and inhibition of proteins in complex cellular systems. However, high-content time lapse microscopy of living cells is still confined to the laboratory and its use is limited to gleaning cellular structure and function. There is a need to scale imaging experiments and methodologies.

1.2. Large-scale imaging of the cell

The concept of high-throughput screening was invented to address the needs of industrial and academic drug development efforts. Fairly basic cellular model systems were used to investigate specific molecular hypotheses. To overcome the limitations of this approach, the concept of phenotypic screening [12] was developed. New emerging developments in (patient derived) *ex vivo* cultures, induced pluripotent stem cells (iPSC) technology, three dimensional (3D) co-culture and organotypic systems hold the potential of designing more disease relevant model systems that will eventually replace traditional cell based models. Horvath et al. [13] recently published a comprehensive review that sets out the principles to facilitate the definition and development of disease relevant assays.

Optical microscopy platforms have evolved to support the growing demand for conducting more complex *in vitro* experiments. Advanced high-throughput microscopy platforms as for example the Cell Voyager 7000S (Yokogawa), Opera Phenix (Perkin Elmer) or the IN Cell Analyzer 6000 (GE Healthcare Life Sciences) offer the capability of acquiring three dimensional (3D) imagery over time. As a result it is now possible to acquire time lapse three dimensional data (3D + T) at scale. Sophisticated software tools help to transform vast amounts of complex multi-channel imaging data into quantitative information.

Thanks to these advances high-throughput cellular imaging is not only used in all stages of target based drug development [15] but is also becoming a relevant tool for investigating more fundamental biological questions. Experiments *in vitro* allow to monitor cell fate, build artifical tumors for studying the link between cancer and inflammation [16,17], and chart the interactions in the microenvironment to new drugs [18–20]. While it is probably

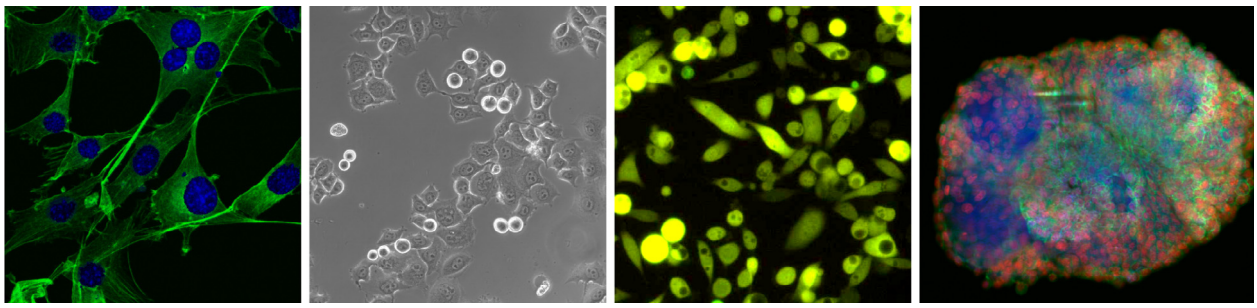

Fig. 1. Biological image examples. These examples illustrate some of the challenges that need to be taken into account when analysing biological imaging data. (Left) shows an image of cytoskeletal and nuclear staining of mouse 3T3 cells. Here it is challenging to assess the precise shape of each cell given the occlusion that occurs. (Middle-Left) This image depicts the artifacts associated with phase-contrast microscopy in the form of clumping which in turn prevents the determination of colonies. While this label free method clearly provides advantages for live cell imaging studies these artifacts make the quantitative analysis more challenging. (Middle-right, Right) These images illustrate that it is not always possible to segment individual cells. The image on the very right shows a spheroid of MCF7 cells in a matrix. The images have been generated in Department of Oncology at the University of Oxford.

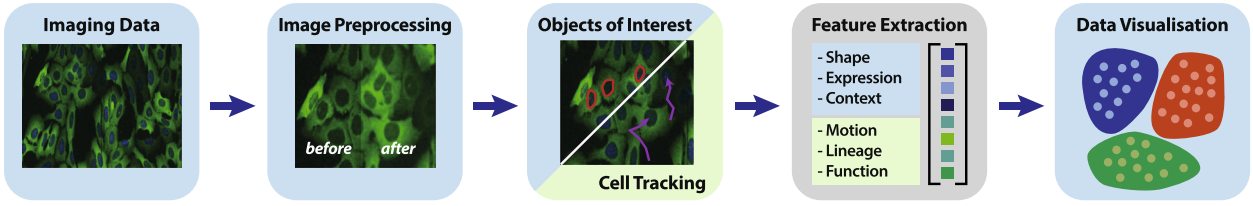

Fig. 2. Imaging based phenotyping. A typical cellular analysis workflow is depicted. An acquired image is processed by correcting for noise and enhancing the signal. Next, cellular objects including nuclei, vasculature, etc. are extracted from the image. If the application demands, cells are tracked and their states are monitored. Features including shape, appearance, and context or trajectory and lineages are gleaned and models are learnt. Finally, the various features are visualized in a phenotypical feature space. This figure also motivates the main section of this review. Image processing methods are being reviewed in Section 2. Algorithms for delineating objects and structures of interest are reviewed in Section 3. Section 4 provides a review of cell tracking methods. The visualisation methods are being discussed in Section 6. For this illustration we used an image out of the set BBBC015v1 provided by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection published in [14].

too early to judge if live cell imaging will have any significant role in clinical practice, it is safe to assume that there is a growing need for processing and analysing images of more complex 2D/3D co-culture models at scale.

1.3. Imaging-based phenotyping

The process of extracting meaningful information from image data (see Fig. 1) is part of an emerging field Houle and collaborators [21] termed phenomics. It is an area of computational biology concerned with the comprehensive high-dimensional measurement of phenomes. Houle et al. [21] distinguish between two different paradigms. The first is to take a large set of measurements at a given point in time, which they refer to as extensive phenotyping. In our context one could extensively characterise a given cell line with a number of different assays. Intensive phenotyping on the other would require characterising a given phenotype in great detail. Imaging specific processes using video microscopy [22,23] would fall into this category. For the purpose of illustration a notional imaging based phenotyping workflow is shown in Fig. 2.

While the difference between intrinsic and extrinsic phenotyping is less relevant to this review, it is the notion of taking measurements that is of fundamental importance. A good measurement should be accurate, robust and precise. Additional key elements [24] are the limit of detection, the response function and specificity. Given that there is noise in the measurement, the limit of detection defines the level below which the response is not meaningful. The response function specifies the dependence of the signal on systematic changes in experimental conditions. Good measurements are necessary for generating reproducible data. The choice of a specific cell segmentation algorithm can, for example, affect the interpretation of an experiment. The systematic analysis of various segmentation methods [25,26] documents how measurement statistics depend on algorithm choices and parameter settings.

The community of computer scientists, engineers and bioinformaticians that develops and advances mathematical methods and algorithms has grown substantially. Biological image analysis is now a broadly recognised area in leading international medical imaging and bioinformatics conferences. A number of challenge competitions, such as the "Particle Tracking Challenge" [27], "Cell Tracking Competition" [28] and Digital Reconstruction of Axonal and Dendritic Morphology Challenge (DIADEM) [29] have been initiated to advance the application specific algorithms. Related challenge competitions can be found on the *grand-challenge.org* web page.

A field which was started by a few enthusiasts (e.g. [30]) has now matured and produces very powerful algorithms that will continue to impact the life sciences. Thanks to fundamental methodological advances in image analysis, signal processing, medical imaging and computer vision the field will continue to evolve rapidly. Examples for one such development are advances in machine learning which will be discussed in this article. We expect that biomedical imaging will become a core part of the life science curriculum. Only with a certain understanding of the underlying methods it is possible to apply these in a thoughtful way to ensure studies produce reproducible results that ultimately help addressing key scientific questions. There is no doubt that microscopy has evolved from a technique of choice for producing stunning and impressive cover images for scientific publications to a technology that turns vast amounts of imaging data into quantitative information.

1.4. Purpose and outline

A number of review articles [2,31–34] have highlighted the opportunities biomedical imaging will provide. The term

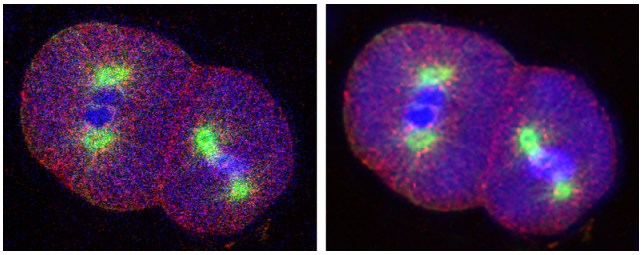

Fig. 3. Image denoising. The example shows the non-redundant interscale wavelet thresholding method developed by Luisier and collaborators. It was specifically developed for Poisson data and presents a less computational intensive methods to other state of the art methods. Figure courtesy of: Florian Luisier, Biomedical Imaging Group, EPFL, Switzerland published in [38].

bio-image informatics evolved and is now used by some. Meijering [35] published a very comprehensive review on cell tracking methods. Dufour et al. [36] provide an overview of assessing 3D morphology. Here we strive to capture the impact more recent developments in computer vision and machine learning will have.

Enabled by machine learning computer vision has emerged as a key technology powering applications ranging from internet search to autonomous driving. The way in which we develop and design algorithms is changing dramatically. Rather than designing such methods from first principles, advanced machine learning techniques now allow us to learn computational models directly from the data. We will discuss how such deep architectures can be used to build reliable algorithms for biological imaging applications.

The workflow shown in Fig. 2 also provides a high level motivation for overall structure of this article. The analysis of shape and motion are the two central themes of this review. Only if we can delineate biological targets accurately, will it be possible to extract a set of meaningful and robust measurements. The segmentation of objects is discussed in Section 3. Methods for cell tracking are presented in Section 4. In the light of recent methodological developments, we believe that these are the areas which will advance most rapidly.

In many cases we cannot work on the raw images directly. Preprocessing methods play a central role in removing image noise and other artefacts. Given the broad range of microscopy methods, Section 2 does not permit a comprehensive review of preprocessing methods. Instead, more recent approaches for processing label-free microscopy images will be discussed.

In Section 5 we discuss some of the available software tools. Rather than limiting the discussion to software relevant to end users, we also provide an overview of toolkits to be used by algorithm developers. With larger data sets information visualisation starts to play a more prominent role. In Section 6 we discuss ideas of visualising the extracted data more effectively. Conclusions and directions for future work are being presented in Section 7.

2. Image preprocessing methods

Shading correction, removal of image noise and the suppression of out of focus light are perhaps the most important steps that would need to be addressed during image preprocessing. Given the focus of this review it is not possible to discuss the necessary calibration procedures that should be part of experimental protocols. Here we only highlight a few topics that should be taken into account during image processing. As the illumination across the field of view will not be uniform an explicit flat-field correction [37] is often necessary. Weak fluorescent staining or short exposure times can result in a low signal-to-noise ratio. In certain practical settings basic noise removal techniques such as median filtering or image smoothing will provide acceptable results.

2.1. Denoising and enhancing signals

A systematic characterisation of the various different noise sources [39] is necessary to achieve best results. More refined signal representations using wavelets [40] capable of providing multiscale representation of images, or signals in general, gave rise to significant advances in de-noising methodologies. The general soft thresholding approach introduced by Donoho [41] needs to be mentioned in this context. Building on this approach, Luisier et al. [38] and Boulanger and colleagues [42] developed noise removal algorithms (see Fig. 3) that are specifically suited for microscopy applications. Additionally, there have been many fundamental contributions to methods that estimate both the background and foreground using non-local and multi-scale methods [43].

Deconvolution is an important and necessary preprocessing step that effects co-localisation analysis and image segmentation [39]. Wallace et al. [44] review the most common deconvolution methods. The problem can also be posed as a regularisation between the observed and an ideal that is modelled using the point spread function (PSF) of the microscope. Campisi and Egiazarian [45] present well-known taxonomy of methods that either systematically estimate the PSF or are blind to the exact form of the PSF.

2.2. Label-free imaging

Label free imaging methods such as phase-contrast, DIC or dark field microscopy provide some clear advantages as they do not require any sample preprocessing. However, processing such images can be challenging. For example, the varying appearance of cell boundaries under phase-contrast microscopy requires special consideration (see also Fig. 1). Su et al. [49] demonstrate that a physics based model allows to extract images features, so called phase retardation features, that enable a more robust processing of the data. An alternative is to apply machine learning techniques (e.g. [48]) for identifying structures such as cellular boundaries. Methods that are capable of extracting more quantitative information from brighfield images continue to improve. Joo et al. [46] developed a technique that can measure minute phase variations caused by changes in refractive index and thickness inside the specimen. Popescu and colleagues [47] propose the diffraction phase microscopy as a new technique for quantitative phase imaging of biological structures. These examples indicate that the collaboration between optical physicists and image analysis experts are necessary before the full promise of these techniques can be realised.

3. Delineating objects of interest

Digital images are represented as pixels associated with various intensity, or brightness values. Biologists however, are interested in objects such as cells, vesicles or tissue components such as blood vessels or glands. Image segmentation allows the identification of object boundaries which then can be used to quantify and analyse various attributes associated the objects of interest.

Despite the development of many segmentation methods over the last five decades image segmentation remains one of the most challenging image analysis tasks [31]. Even relatively minor changes in imaging conditions can require algorithm or parameter re-optimisation. Firstly, segmenting cellular images requires the identification of multiple objects in the image. Cells have heterogeneous shapes that are typically subject to dynamic changes. It is

therefore difficult to define shape in the form of mathematical models. Secondly, cell compartmentalization as well as intra- and inter-cell variability induces non–homogeneous marker distributions within and across cells, leading to undesirable image features such as intensity gradients. When analysing dense cell populations it is often very hard to assign features. While many splitting criteria for segmenting touching cells have been proposed they often only hold in specific settings. Unless a concrete objective or application context is given, the task of image segmentation is not a well defined problem.

In certain cases pixel based similarity measures are already sufficient for identifying objects of interest. One intuitive example is the identification of DAPI stained nuclei. Generally, purely data driven approaches are not sufficient for robust object delineation. Here, it is necessary to incorporate prior information through user interaction or mathematical models which are either designed manually or learnt from example data.

In many biological applications vast amounts of data are being generated and it is not feasible to rely on user input for the purpose of segmenting individual cells. Instead, it is necessary to design fully automated algorithms that are robust to changes in imaging conditions. Recent developments in computer vision and machine learning have helped in making significant progress towards this goal. In this review, more traditional methods are being referenced to point out what challenges need to be overcome. The remainder of this section focuses on more recent developments that will greatly impact our ability of developing robust algorithms to effectively process large data sets.

A discussion of traditional methods in Section 3.1 provides some historical context. These algorithms are now core components of most software packages (see Section 5) and are still used in many applications. Although there is no systematic theory of image segmentation, the concept of partitioning an image into segments by the means of clustering allows to present some of the more recent developments in a consistent fashion [50]. Modelling the image probabilistically is another important concept. Section 3.3 outlines how spatial relationships between pixels in the image can be modelled. This allows us to consider not only texture but also membrane boundaries which play an important role for delineating objects. Finally, we discuss some of the opportunities the emerging body of deep learning will offer. They provide an entirely different approach for learning computational models directly from imaging data but require a large set of annotated training images.

3.1. Traditional approaches

Many of the traditional image processing and computer vision methods [51] were developed for processing and analysing binary images. Meijering [52] provides a comprehensive review of the development of segmentation methods for biological imaging applications. Given that the basis for fluorescent microscopy is the use of differently coloured labels such images can be easily converted into binary images. At first sight, basic automated thresholding techniques (e.g [53]) combined with suitable pre-processing and post-processing appear to be a adequate solution. However, touching cells, image noise, inhomogeneous staining and uneven illumination are typical causes for segmentation errors. The field of mathematical morphology [54] was developed for analysing geometrical structures, based on set theory, lattice theory, topology and random functions. The well known watershed segmentation algorithm has been developed in this context. Watershed segmentations have been applied with considerable success to biomedical imaging [55–57]. The quality of watershed segmentations depends on selected seed points as well as suitable image pre-processing steps. Generally, relatively minor changes in imaging conditions require further algorithm optimisation.

A recent review [58] highlights the impact active contour models continue to have on biological imaging. In general an active contour is a curve that evolves from some initial position towards the object of interest, a biological target. The contour evolution is governed by an energy function, called the snake energy. While we cannot provide a comprehensive review of the work on active contours, it should be mentioned that the development can be split into three broader topics. Point snakes denote the first category. Nominally, in active contour models [59] the curve is defined on the discrete pixel grid. The resulting representation unfortunately contains many parameters making it difficult to achieve robust performance. Based on the mathematical concept of implicit functions the idea of geodesic snakes [60–62] follows a more principled approach. The objective function captured by *snake energy* effectively controls the smoothness of the resulting contour and the model can deal with topological changes, which allows to segment highly complex objects. Unless constrained correctly geodesic contours can lead to overfitting. The optimisation process tends to be computationally expensive. Parametric snakes introduced through the work by Staib and Duncan [63] employ a parametric representation (e.g. B-splines) of the contour. As a result it is possible to design very fast algorithms which effectively incorporate shape priors. The idea of the snakuscule [64], a minuscule snake, takes this idea to an extreme. It enforces a circular shape and can be used for applications such as cell counting and vessel extraction. The use of active contour models is not limited to image segmentation. By initialising the contour on one image of a time lapse sequence and optimising it on the subsequent image, this method has been used effectively for cell tracking. Details will be discussed in Section 4.

3.2. Segmentation through clustering

The idea of grouping a set of pixels based on colour or local texture is a paradigm that is the basis of many image segmentation algorithms. The most basic example is the separation of background and foreground pixels. Various forms of clustering (see for example [66]) have been explored for generating image segments which are consistent in colour and texture. For characterising textures [67–69] and other structures [70,71] (e.g. dots of vessels) a vast number of low level image features have been developed. Methods that label each pixel site independently will not produce locally consistent image segments. The concept of superpixels addresses this problem by defining local clusters and limiting the search regions for assigning new pixels to a given region. One of the most successful methods proposed by Achanta et al. [65] reliably identifies regions which adhere well to object

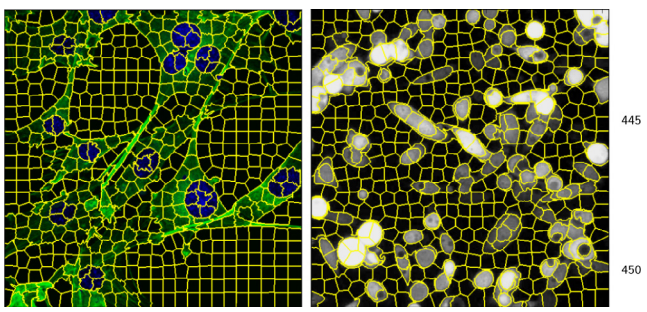

Fig. 4. Superpixels. Superpixels provide a simple and effective method of generating locally consistent patches. The SLIC superpixel method [65] is used to illustrate the efficacy of this approach. Both images are results of the SLIC method after being deployed on images in Fig. 1. A set of 600 superpixels segment the images. This approach can also be used to make the processing of very large images more efficient.

boundaries and are efficient to compute. The examples shown in Fig. 4 illustrate that additional grouping is necessary before meaningful objects can be identified.

Similarities between pixel sites can be captured in form of an affinity matrix *A*. The set of pixel sites *V* together with the matrix can be used to define a graph *G*. The graph can now be cut into connected components that have relatively large interior weights by removing edges with low weights. Normalised cuts [72] segments an image by measuring the total dissimilarity between groups of pixels as well as the dissimilarity within groups. Cour et al. [73] provide a formulation that takes the edge information in an image into account. The iCut algorithm [74] illustrates how this approach can be applied successfully to biological imaging. As images grow in size the affinity matrices become prohibitively large. Defining the graph on a set of superpixels instead of the original pixel grid of the image is one approach to avoid this problem. Finally, a method inspired from the tracking literature has also demonstrated much practice. In [75], the graph cut algorithm is used in the context of tracking cells from image plane to image plane.

Yet another hybrid method was proposed in [76] where several methods were assembled to address the problem of segmentation of large microscopy images. Level sets, the geometry-inspired centroidal Voronoi tessellation (and *K*-Means), and local estimation of pixel intensity and texture were deployed in tandem to create 2D and 3D segmentations.

3.3. Probabilistic segmentation

By modelling the value at a given pixel as a random variable we can model images probabilistically. As images contain rich structure locally, the value of a given pixel depends on it's neighbours. Markov Random Fields (MRFs) provide a formal framework for modelling complex probability distributions in form of a graph. Each node in the graph is associated with a random variable. Interactions between random variables are specified through edges in this graph. Such distributions are of often expressed in terms of energy functions and clique potentials. Geman and Geman [77] originally introduced a MRF for reconstructing noisy images. Since then MRFs have been applied for a broad range of computer vision applications including image segmentation. Determining the maximum a posteriori estimate of such a model is an *NP-hard* problem. The originally proposed stochastic optimisation techniques are computationally expensive and slow.

By proposing a new class of energy minimisation algorithms based on graph cuts Boykov et al. [78,79] enabled the application of MRFs for a broad range of computer vision and image analysis task. This approach led not only to fast interactive segmentation methods for medical imaging [80], it also stimulated a number of successful segmentation methods for biological applications.

Building on the idea of graph cut based active contours [81] Chen and collaborators [82] developed a segmentation approach that is particularly suited by RNAi screens. Here, the information of different fluorescent channels is combined to obtain better object boundaries to aid image segmentation. The pattern-based cell segmentation approach proposed by Dimopoulos et al. [83] is fixated on the detection of membrane patterns; the approach effectively segments densely packed cells in an accurate manner. Probabilistic segmentation methods have been leveraged by Mosaliganti et al. [76] to enforce separation of overlapping cells.

3.4. Learning models from image data

Methods of machine learning play a vital role in a number of tasks including feature selection, classification and the discovery of latent structures. More recent developments in applying deep learning [84] to computer vision promise to have a very fundamental impact on how we build and design algorithms for analysing biological image data. The fact that neural networks can be used to approximate almost any continuous function has been known for a while [85]. This theoretical result [86] even holds if the network consists of only a single intermediate layer between input and output neurons. While it illustrates the universal applicability of the approach, it does not provide any practical guidance for designing neural networks for specific problems.

Recently proposed convolutional networks for object recognition [87,88] and semantic segmentation [89] not only demonstrate that such approaches can outperform traditional methods, they also illustrate that it is possible to learn such models directly from raw image data. Data preprocessing, feature extraction and segmentation which were considered to be different steps in a traditional image analysis pipeline are now all integrated into one holistic computational model. Hence image analysis pipelines no longer depend on a set of user defined features.

As layers can be fully connected these models explore a richer set of spatial interactions across the scales when compared to traditional MRFs. The price for this level of complexity is the fact that millions of free parameters need to be estimated from labelled training images. One solution is to train the model on a large corpus of natural images and then use transfer learning [90] to retarget the model onto a specific data set. Further, training models on simulated data [91] have shown extremely promising results. Here it would be possible to utilise earlier generative models for microscopy data [92,93].

Given the rapid development of the field, it is difficult to provide a comprehensive overview of deep learning to life science applications. Ning et al. [94] developed a automated phenotyping approach for C. elegans embryos obtained through DIC microscopy. A convolutional network is used to map raw pixels into output labels representing cell nuclei, nuclear membranes, cytoplasm and cell walls, and extracellular medium. Ciresian and collaborators [95] train a network for detecting membranes in electron microscopy data. Using data from the ISBI 2012 EM Segmentation Challenge they demonstrate that their approach outperforms competing techniques by a large margin with respect to different error metrics. One of the best scoring approaches in the Mitosis Detection Algorithms 2013 (AMIDA13) challenge included a convolutional network based architecture [96]. Ronneberger et al. [97] demonstrated how the same fully connected convolutional networks [98] can be applied towards the segmentation of electron microscopy and differential interference contrast imagery. Similarly, during the recently held CAMELYON challenge [99], which aims to identify histology pattern that are associated with metastatic breast cancer, many of the successful entries explored the use of CNNs and related recurring neural networks.

3.5. Open challenges

While these most recent advances open very exciting opportunities, we also need to advance methodologies that help to extract reliable data from imperfect segmentations. Overlapping and partially occluded cells will continue to cause uncertainty that even an apparently perfect segmentation approach will not be able to resolve. This problem will continue to confound the research community given the complications of occlusion that will arise when 3D objects are projected onto 2D imaging planes.

Mosaliganti and collaborators [76] present one approach where constraints are imposed based on ideal separation of nuclei. Subsequently, an objective function is optimised to determine the necessary separating plane. Another option is to discard data that does not satisfy certain assumptions. Meaburn et al. [101] present a systematic analysis of genome reorganization events during early tumorigenesis. Here a multi-stage classification [57] is being

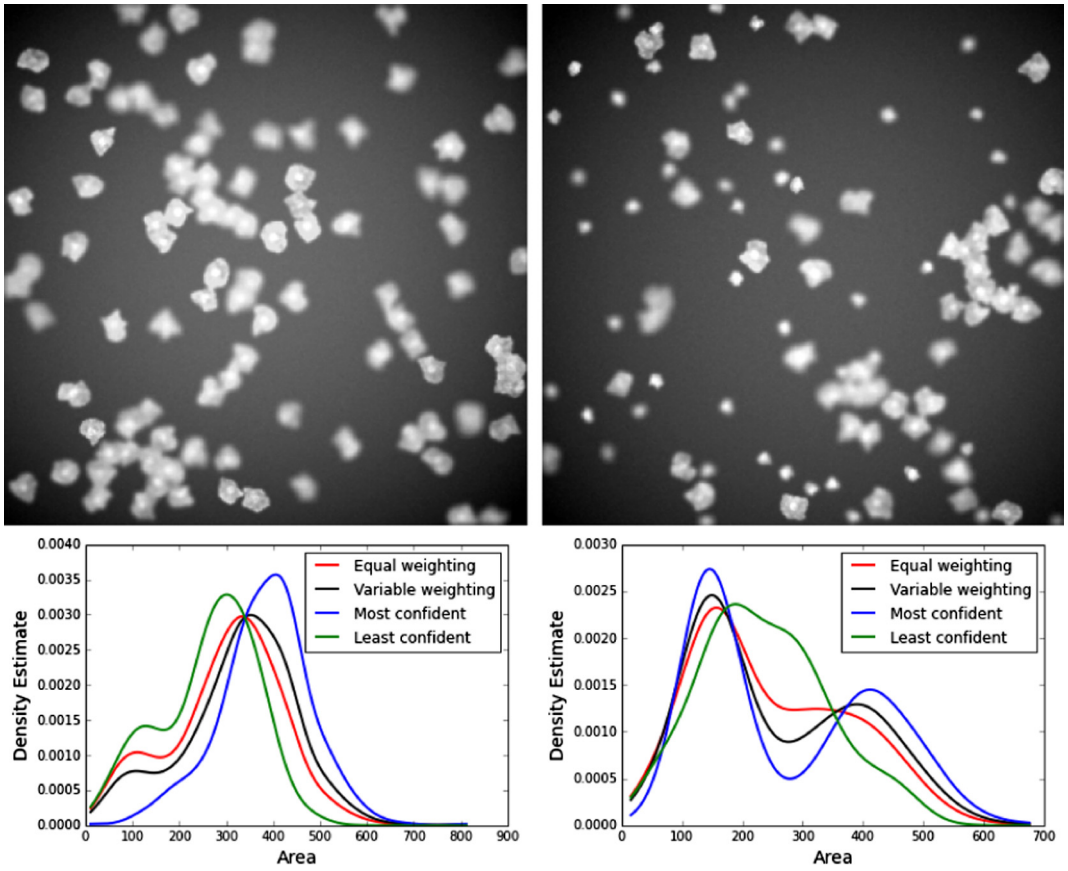

Fig. 5. Accounting for uncertainty in segmentation. Nketia et al. estimate a confidence score for each of the identified candidate regions. This confidence score is then used to generate an overall estimate. Here the approach is illustrated with the help of two simulated images. The image on the left depicts a mono culture and the one on the right a mixed cell population. A comparison of the resulting distributions is shown below. Figure courtesy of: Thomas Nketia, Institute of Biomedical Engineering, University of Oxford published in [100].

applied to select a set of cells for analysis. Alternatively the inherent uncertainty of image segmentation can be taken into account. Nketia et al. [102] propose to estimate the confidence of each identified object and incorporate this confidence score in the measurement statistic with the help of a kernel density estimate [103]. An example on some simulated date is shown in Fig. 5. Further work is necessary to establish a more robust confidence metric for the resulting measurements.

In certain cases it is not necessary to segment the image. Arteta [104] developed a learning based framework that is designed to count cells in the presence of overlap. Here dot annotations are used to capture the required input from users. This approach has been extended to quantify Drosophila egg laying [105]. The accuracy of the original counting method is increased through correction of bias observed in the algorithm output.

Recently Ulmann et al. [106] also propose an interesting approach for a segmentation-free classification of images. Here over 900 features are being extracted from the image but only a subset of those accounting for 98 % of the variation are used for analysis. A linear discriminant analysis is used for classification. The method has been tested with success on the Broad Bioimage Benchmark Collection and data from the Human Protein Atlas [107].

While machine learning methods enhance our general ability for designing more robust algorithms, further work is needed to determine what type of preprocessing is required to ensure that these methods can be applied in a general setting. Here it might be possible to build on a set of tools that has been developed for processing histology images [108,109].

4. Cell tracking

Cell tracking is essential in understanding the temporal dynamics of cell behaviour in time-lapse sequences. Cellular density in time lapse sequences obtained from typical biological experiments often tend to be high given the very closely-packed arrangement of cells in most frames. In addition, equipment and biological limitations including risk of photodamage reduce the frequency at which images can be captured over time. Consequently, this results in time lapse data with significantly low temporal resolution compared to that of conventional video. As a result, motile cells in the time series data seem to jump in a random fashion between consecutive frames showing minimal spatial overlap. These artefacts make cell tracking more complex and challenging than conventional object tracking in video [110]. The difficulties are further compounded by sudden changes in cell morphology over time [111].

Current methods for cell tracking build on the development of visual tracking algorithms for more general computer vision applications [112]. They can be broadly categorized into deformable models, state space models, and segmentation-based object association. In Section 3.1 deformable models were discussed in the context of image segmentation.

Deformable models involve a contour evolution approach, usually a level set or an active contour to obtain the boundary of an object in the current frame by evolving the contour from the previous frame [113–116]. In practice, it involves initializing the segmentation of objects in the first frame and updating in subsequent frames. Such contour evolution models fail in applications where

objects undergo sufficiently large displacements or show little overlap between consecutive frames [117]. These approaches also fail to detect cells that enter the field of view. Li et al. [115] address this by treating new cells using a local association scheme.

State space models for tracking often employ stochastic filtering techniques that rely on the underlying motion or appearance model of the tracked object. In this regard, the model expects the object to follow some assumed motion pattern but does not require an accurate segmentation of the tracked objects. They tend to be computationally demanding due to the large number of hypotheses pertaining to the motion-under-scrutiny and yet allow for complex observation models [118]. State space models are, however, better suited to handle larger displacements [119,120].

Segmentation based object association tracking models involve two major processes: object detection or segmentation in all frames and the association of objects in different frames to obtain a lineage. This allows cell tracking to be handled as two separate tasks; detection and association. Such association methods have been shown to be effective for cell tracking, to scale well and also achieve high accuracy in overall cell tracking [117,121–125]. The segmentation step varies widely depending on application or imaging modality.

Another method that could be loosely associated with the object association model is template matching with image registration between time points. The registration could be applied at the pixel level [126], at feature points [127], or for entire cellular objects [128]. The remainder of this section will focus on various approaches used in object association models as these have been most effective for cell tracking in time lapse phase contrast data [125,123,124]. Magnusson et al. [129] use the Viterbi algorithm to achieve a globally consistent cell tracks.

4.1. Learning-based cell tracking

Tracking by assignment usually involves optimization features or objective functions which need to be adjusted for obtaining good assignments. Such parameters could be learned from training data by casting tracking as a learning problem [117]. Such methods have been applied to tracking pedestrians in conventional video with models derived from the Conditional Random Field (CRF) [130] and HybridBoost [131] techniques. The main challenge with learning based methods is obtaining accurate training data which usually involves costly annotations. In this regard, a cell labelling and active learning [132] have been applied to minimize the cost of annotation that is required in [117].

Lou and Hamprecht [117] present a more comprehensive learning-based formulation of the minimum cost flow theory method proposed by Padfield et al. [123] which is purposely built for cell tracking. The underlying model accounts for mitosis, appearance and disappearance of cells.

The approach *learns* the parameters associated with tracking events from training data to improve robustness and eliminate parameter tweaking. The learning of parameters hence provides a more robust approach to estimating model parameters. Such a learning-based approach however requires ground truth training data that contains frame-to-frame association pairs of all transition events for all cells. In the standard frame transition events, a cell may *move* or *divide*, and can *appear* or *disappear* from a frame. Accounting for appearing or disappearing cells is important as cells near the boundary of the field of view of the camera could move out and in depending on the direction of motion of the cells. Also, by introducing additional events *merge* and *split*, Lou et al. [117] account for the under-segmentation and over-segmentation errors typically observed in the detection step of closely-packed objects.

4.2. Global object association tracking

As indicated earlier, the object association methods have shown to be effective in time-lapse. However, the challenge of accurately accounting for cells that leave or appear in a frame as well seeming merges and splits over many consecutive frames still remains. To resolve this, Bise et al. [124] proposes a global object association model rather than the local association methods involving association between consecutive frames only as in [123,117].

Global association methods associate objects over multiple frames by joining multi-frame trajectories. This could be achieved via *tracklet stitching* [133,134]. This involves first generating reliable fragments of object tracks (tracklets) and then merging these tracklets into more complete tracks. Connecting tracklets is achieved in [133] via the Hungarian algorithm [135] and by dynamic programming in [136].

The global association methods mentioned above are built for general object tracking and do not address specific cell behaviour such as cell division. The formulation proposed by Bise et al. [124] however accounts for division and possible cell segmentation errors. The tracklet association problem is formulated as a maximum-a posteriori (MAP) problem and solved by linear programming to obtain cell trajectories and lineage trees.

4.3. Open challenges

Cell tracking methods have already enabled large scale biological studies that were previously not possible. The *Mitocheck consortium* [137] processed more then 190,000 time lapse movies of fluorescently labelled HeLa cells providing records of over 19 million cell divisions. This data was utilised to provide time resolved profiles of RNAi induced loss-of-function phenotypes resulting from siRNAs targeting the entire genome.

To study multi-generational differences between cerebral cortex neural progenitor cells Winter et al. [138] use phase contrast microscopy allowing image capture at a temporal resolution sufficient for accurate tracking through multiple rounds of cell division in a label-free manner. The cell tracking tool *Lever* [139] became the foundation of an entire research programme.

Extending these tracking approaches for analysing complex cellular behaviours is an open challenge. While the analysis of cell cycle phase [137,140,141] has been explored, other complex phenomena such as for example the various cellular events leading to cell death or collective cell migration have not been addressed. Another open an unexplored topic is also the multi-generational cellular response to drug compounds.

5. Software tools

Eliceiri et al. [33] provide a comprehensive overview of all the different software tools that are necessary for the implementation of an image informatics workflow. Here image acquisition, storage, analysis as well as image and data visualisation need to be taken into account. This section focuses on freely available open-source software tools. The following three sections provide an overview of software packages for end users, tool kits facilitating algorithm development and client server based environments. Open challenges and directions for future developments will be discussed in the final section.

5.1. Supporting end users

Intuitive and well-supported software tools play a crucial role of providing end users access to this technology. Today, users can choose between very mature and sophisticated software tools.

Furthermore, users also benefit from support through tutorials and workshops which are provided by the developers themselves as well as through initiatives such as the Euro-BioImaging available on the website www.eurobioimaging.eu and the Global-BioImaging project.

Undoubtedly ImageJ and the recently enhanced distribution of the same software, *Fiji* [142], are the oldest and most widely used tools for scientific image analysis. The software can be extended with help of a large number of plug-ins ranging from cell counting to co-localisation analysis. From the quick assessment of image quality to the scripted analysis of smaller image data sets it can be used for a wide range of tasks. As it is written in the language *Java* it can be easily deployed on all popular operating systems. While very versatile, some of the more advanced algorithms for segmentation and tracking are not available. It is probably less suited for the routine analysis of large data sets.

Cellprofiler [143] has been developed to facilitate the interactive data exploration, analysis, and classification of large biological image sets. Originally written in *MATLAB*, the software is now implemented the computer language *Python* and includes a number of powerful features including a range of machine learning tools. *Cellprofiler* provides an open source alternative to commercially available high-content image analysis software packages such as *Cellomics* (ThermoFisher Scientific), *Harmony* (Perkin Elmer) or *IN Cell Investigator* (GE Healthcare Life Sciences). As an open source tool it provides the advantage that it can be extended very easily. However compared to other commercial tools it is not very well integrated with high-throughput microscopy hardware.

While *Cellprofiler* [144] is more geared towards 2D high-throughput screening data, *Icy* [144] provides a very comprehensive set of tools for the analysis of multi-channel 3D images. Developed and maintained by the biomedical image analysis group at the Institut Pasteur (Paris, France) it builds on a number of existing open source libraries and can also be extended through a broad range of plugins. The *ilastic* [145] platform is a simple and user-friendly tool for interactive image classification, segmentation and analysis. Notably, it provides access to some very advanced machine learning based algorithms for segmentation and tracking.

5.2. Supporting algorithm developers

Engineers and computer scientists rely on sophisticated software libraries and toolkits. These software tools provide important core capabilities which include the reading of application specific file formats and access to previously developed technology. In some sense they form the bases of extending the capabilities of the software packages discussed previously.

The National Library of Medicine *Insight Segmentation and Registration Toolkit (ITK)* [146,147] is the most widely used open source medical image analysis toolkit. It is designed to support *N*-dimensional images. Still, working and extending the *C++* based libraries does require training. However, as the algorithms that have been included in the toolkit have been carefully validated, *ITK* made an invaluable contribution to the scientific community. *SimpleITK* [148] now provides a scripting interface to the underlying libraries, hence making them accessible in languages such as Python and R. The newest release now includes access to the registration algorithms which will be of interest to a number of groups. *Farsight* [149,150], which is a more specialised set of tools for the analysis of multi-channel fluorescent microscopy images is built directly on *ITK*.

Scripting languages not only provide the advantage of fast prototyping, they also allow the systematic integration of well established libraries for numerical computing, linear optimisation and now machine learning. With the increase in data volume it will be necessary to process data sets on dedicated compute clusters. *Python* based environments allow one to make this transition. Possible performance bottlenecks can be eliminated through targeted optimisation. *Python* is increasingly viewed as the *lingua franca* of data analytics. It provides a great environment for developing analysis scripts that can be deployed on end user as well as specialised compute clusters. Rather than entering into a debate whether or not is should be used instead of statistics package based on the language *R*, we argue that these two languages, which are both distributed under open source licences, complement each other. With the *Python* interface of *OpenCV* [151] and the scikit-image libraries [152] *Python* provides a very rich set of image analysis tools. To date there is a lack of libraries that address microscopy specific analysis solutions. The *Python Microscopy Environment (PyME)* [153] is being developed to address this gap. It is targeted to high-resolution microscopy. In addition, all the underlying software modules provided by *Cellprofiler* can be accessed as *Python* libraries. *SimpleITK* can now also be installed directly as part of leading *Python* distributions. *Python* also provides access to popular deep learning libraries such as *TensorFlow* [154,155] and *Theano* [155].

5.3. Creating integrated environments

As data sets grow larger in size it is often no longer feasible to analyse the experimental data on a personal workstation or laptop. Environments such as *OMERO* [156] and *Bisque* [157], which were originally developed for storing and retrieving microscopy image data sets have now been extended to provide client–server software the visualisation, analysis and management of microscope images. Specific expertise and hardware is necessary for the setup and maintenance of these environments. Currently such capabilities will be limited to industrial laboratories and larger centres as they have the capacity to maintain and customise these solutions.

5.4. Open challenges

Today, it can still be difficult to reproduce image analysis experiments. A change in implementation details or parameter settings might actually result in different results. Provenance based methods are certainly par for the course. Further, standardization will be required to establish norms for analysis and discovery. It will be necessary to develop publications standards that are similar to those established for microarray analysis [158,159]. Furthermore the tools for processing large data sets need to be improved. What is already true for the processing of sequencing data today, will very likely become a reality for many future imaging studies. The necessary data will have to be hosted on large databases and will have to be processed on dedicated compute clusters. More intuitive and user friendly interfaces will have to be developed. In addition various analysis and visualisation capabilities will have to be integrated. Substantial further work will be needed to achieve this goal. But one day users who analyse their images using their favorite tools will routinely process large image collections on server hosted environments.

6. Information visualisation

Image analysis is only the first step in imaging studies that results in large and complex high dimensional datasets describing the phenomena-of-interest or the differences between biological samples. Visualisation plays an important role in understanding and analysing complex microscopy data. Dedicated software is needed to effectively view and inspect 3D time-lapse data that has been acquired in multiple channels. Segmentation and tracking results need to be displayed in context of the original data to

support algorithm development and analysis. Furthermore visualisation enables the identification of interesting phenotypes or artefacts, and the development statistical models of cellular shape and appearance. The effective visualisation of these datasets is crucial for identifying possible relationships and hypothesis in the data and choosing appropriate statistical or mathematical modelling methods.

Imaging information contributes to building quantitative models of the cell and cellular function. Including a detailed discussion of these methods is outside the scope of this review article. The work of Murphy and collaborators [160] and the Virtual Cell Project [161] serve as excellent examples for such efforts. In this context the recently NIH funded 4D Nucleome project should also be mentioned. It aims to advance our understanding of the principles underlying nuclear organization in space and time.

6.1. Viewing complex imaging data sets

Many tools have been developed to allow efficient exploration of complex imaging datasets from different angles. Walter et al. [162] provide a very comprehensive and broad overview of different visualisation methods for images. For example, *Icy* provides synchronised viewers to allow inspecting different time-points or locations of the image simultaneously [144]. Furthermore, the results of image analysis can be overlaid on raw images which enable validation of the analysis methods. Another useful tool for exploring large and complex 3*D* imaging data is *Volume3D* which utilises surface rendering to allow real-time visualisation of gigabytes-sized 3*D* imaging datasets on a typical laptop or a personal computer[163]. *Clear Volume* [164] is a dedicated open-source tool for visualising light sheet microscopy. The tools enables viewing of the data during image acquisition. 3*D* image data can also be streamed over the internet for remote viewing. *CellProfiler Tracer* allows visualisation of time-lapse data and exploration of the resulting cell trajectories, lineage tree as well as the progression of selected cells along time simultaneously [165]. These different tools aim to facilitate quantification of phenotypes in separate imaging datasets.

6.2. Shape and appearance variation

One goal of cell biology is to understand how cells adopt different shapes and expression profiles in response to varying environmental and cellular conditions. A statistical analysis of the quantitative data derived from imaging studies is one first step towards advancing our understanding on how molecular processes govern cellular morphology. Here we need to differentiate between desciminative and generative models. The PhenoRipper software [167] was developed in the Altschuler and Wu laboratory with the aim to enable the rapid exploration of high-content microscopy images. PhenoRipper permits comparison of images obtained under different experimental conditions based on image phenotype similarity. Because the software analyses the images on a set of blocks rather than individual cells the analysis does not rely on an explicit cell segmentation method. The set of features is selected automatically. As a result of this analysis the software will map the data into an high-dimensional feature space and identify a number of classes. The benefit of this approach is that it hides the details of the machine learning procedure from the user.

Generative approaches can help to relate the features to interpretable visual models. Sailem and collaborators [168] present an approach for modelling cellular shape. Based on image derived features they employ Gaussian mixture modelling and hierarchical clustering for developing a graphical model that explaines how the cross talk between Rac and Rho contribute to shape changes in wild-type Drosophila BG-2 cells. By formulating a transport based framework Wang et al. [169] develop a generative approach for modelling cellular shape and appearance directly in the image space. The transport based framework allows to address several tasks such as discriminating nuclear chromatin patterns in cancer cells, decoding differences in facial expressions, galaxy morphologies, as well as sub cellular protein distributions. It is for example possible to learn a visual dictionary exemplars and then compare the instances of this with new data points. In order to improve the representation an appearance manifold Sing et al. [170] learn a distance metric from labelled data. This metric is locally adaptive to account for heterogeneity in the data. This approach allows to analyse the heterogeneous expression patters in cell nuclei.

6.3. Visualising phenotypic structures

The development of visualisation tools to address the nature of image-based datasets lags behind and is still largely restricted to the traditional plotting toolbox that includes scatter plots, parallel coordinates, and heat maps [171]. Visual analytics tools combine different traditional visualisation methods to enable the user to

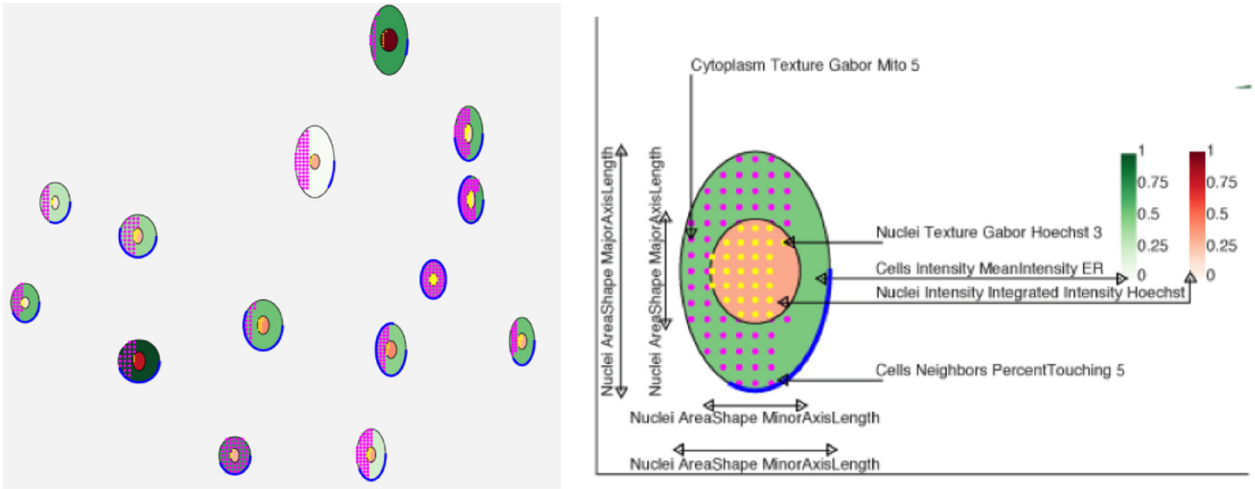

Fig. 6. PhenoPlots phenotypic measurements. Phenoplots of 9 phenotypic measurements of the Human Bone Osteosarcoma Epithelial Cells from the *U2OS* cell line are shown. The position of cells reflects their X and Y coordinates. Image derived phenotypically relevant measurements are being mapped into pictorial structures to provide a more intuitive visualistion. This approach allows to visualise a set of phenotypic traits in form of a compact illustration. Mito: mitochondria, ER: Endoplasmic Reticulum. Figure courtesy of: Heba Sailem while at Insitute of Cancer Research, London published in [166].

interact with the data in order to drill down or zoom up different views of the data simultaneously [172,173]. Recently, few methods have been developed to address the specific needs in large biological datasets. For example, *glyph SPLOMs* allow efficient exploration of the associations and dependencies between tens of variables by representing dependency relationships as a certain number symbolic categories [174].

Another interesting method is *PhenoPlot* that provides pictorial elements to intuitively and quantitatively depict underlying phenotypic structures [166]. Such intuitive representations greatly facilitate interpreting the information in high dimension and easily relating it to the measured structures. The *PhenoPlot* representation of 9 variables describing the shape, texture, and intensity of different organelles in a given image of Human Bone Osteosarcoma Epithelial Cells from the *U2OS* cell lines [175] is shown in Fig. 6. Taylor and Noble [176] developed an approach for the interactive exploration of large image data sets. Here user generated measurements are utilised to arrange images virtual light table. This way the user can effectively associate images with meta data.

7. Conclusion & future directions

In recent years the field of biological imaging has grown significantly. The community has developed new approaches and tools that have become an integrative part of biological studies. Algorithms and methodologies continue to evolve. The recent advances in machine learning and computer vision, which allow learning capable and robust algorithms with the aid of deep learning directly from the data, have been highlighted in this review. Rather than setting up pipelines that utilise a number of algorithmic components, users can now learn new or adapt pre-trained models for certain tasks. Importantly, such an approach could reduce the number of parameters that would need to be determined by a user.

Increasingly, it will become necessary to process very large image data sets. Perhaps these should not be confused with the overused term *big data*. However, these data sets are sufficiently large to require dedicated server based solutions. Rather than struggling to process increasing large data sets on their own computers, users will store and process their data on dedicated compute clusters. Principles from bioinformatics will have to be adopted for allowing users to deal with such data sets in a transparent fashion.

The systematic integration of quantitative image analysis with mathematical models to obtain more biophysically relevant measurements is another important new research area. For example, Olivo-Marin and collaborators [177,178] propose an novel approach for measuring pressure and forces in the cell. Here the intracellular material is being represented as a 2D incompressible fluid and this model then acts as a control parameter for solving the classical optical flow equations. In general image derived data could be used for providing more realistic initial conditions for a mathematical model. In turn the underlying model could act as a prior for the extraction of image derived measurements. It is our contention that bioimage analysis and informatics will continue to play a very important role in gaining insights into the functioning and maintenance of living cells way beyond the relatively simple observations and methods of the early pioneers like van Leeuwenhoek.

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