Article:
Gokhale, P.J. (2016) Extracting information from imaging cytometry: a review. Biotechnic and Histochemistry, 91 (8). pp. 540-548. ISSN: 1052-0295

https://doi.org/10.1080/10520295.2016.1247987

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Imaging cytometry

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Extracting information from imaging cytometry: a review

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Abstract

The extraction of statistically meaningful quantitative information from microscopy images is increasingly important for modern biological research. Obtaining accurate, quantitative information from biological specimens, however, is a complex process that requires optimization of several parameters. One must consider the number of probes, fluorescent channels required, type of plate to be used, number of fields to be acquired and optimal resolution for image acquisition. The extraction of information from images is dependent on and can be aided greatly by careful consideration of the factors involved in the image acquisition process. I summarize here the general principles behind the imaging and software technology that is used to quantify images and highlight particular issues of concern for critically applying image quantitation techniques for research.

Key words: cytometry, high-content analysis, high-content imaging, microscopy, review

Imaging cytometry is the extraction of quantitative information from images that usually are obtained by microscopy. The images can be bright field or multi-channel fluorescent images. Most readers presumably are familiar with flow cytometry, which is the most common method for obtaining quantitative information from single cells (Jahan-Tigh 2012). Flow cytometry involves identifying single cells that are stained with fluorescent probes by measuring the intensity of fluorescent signals. Imaging cytometry can be thought of as a comparable method, except that it uses cellular imaging to obtain information about cells. Imaging cytometry has become widespread following the adoption of automated modules for conventional microscopes and the availability of integrated high-content screening (HCS) instruments. HCS instruments comprise all the modules associated with an automated microscope assembled into a single package that allows relatively easy acquisition of thousands of images under many conditions.

In general, an object, be it cells, tissue sections etc. that can be stained and visualized can be analysed using imaging cytometry to extract quantitative information. The most common format that is used in imaging cytometry relies on cells deposited in multi-well plates. The use of multi-well plates allows researchers the flexibility to include in a single experimental setup relevant

controls and multiple experimental conditions. Multi-well plates are available in a wide variety formats ranging from single wells through to high-density plates containing 1536 wells.

Instrumentation for imaging cytometry

General setup

In principle, any set of images from a wide variety of instruments can be used for analysis but careful matching of instrumentation to the reagents and experimental setup can often enable the optimization of results.

A conventional (non-automated) microscope setup can be used for simple analyses. Pictures are acquired manually and can be loaded into suitable software for analysis. As either the number of objects (cells etc.) or conditions increases, manual acquisition of images becomes increasingly problematic. Automation options for conventional setups offer the ability to scan multiple positions using automated high-precision x–y stages and to maintain focus using so-called hardware autofocus devices that can compensate for focus drift caused by thermal changes, plate/slide irregularities etc. When coupled to automated filter wheels and

shutters, these systems offer scalable automated multichannel image capture that is the basis of proper quantitative analysis.

Automated high-content imaging (HCI) systems are “microscopes in a box,” where the system is optimized for ease of use. Optical components have been assembled with automation and user-friendly control software to create systems that do not require specialized knowledge to assemble and operate. A disadvantage of the HCI systems is that they are not modular. After purchase, they can be altered in limited ways, e.g., by changing filters and polychroic mirrors.

Whether adapting a conventional laboratory microscope system or purchasing a high-end HCI system, the quality of the images is the critical factor for obtaining accurate quantitative information. So what features affect the instrument's ability to obtain high quality quantifiable images and to perform analyses?

Light sources for HCI

Microscopes can use three different types of light source: conventional lamps, lasers and light-emitting diodes (LED). Traditionally, wide field microscopes used mercury, xenon, or more recently, metal halide lamps for illumination. Lamp-based light sources offer a broad range of excitation wavelengths, but also can emit substantially at infrared wavelengths, which can result in heat transfer into the

microscope body. Consequent thermal expansion can affect the microscope's ability to hold focus and requires systems to be prepared and warmed well before imaging is undertaken to stabilize the entire system. Mercury discharge lamps are not usually suitable for quantitative imaging owing to the spikes in emission output that occur at certain frequencies. An additional practical issue is that both xenon and mercury lamps suffer from short lifetimes, typically 100−200 h for mercury and approximately 1000 h for xenon. This can result in considerable expense when systems are used to take thousands of images on a regular basis. To overcome these problems, modern microscopes are fitted with either solid-LED light sources or with several lasers.

Lasers provide powerful monochromatic light sources, but only over a limited set of wavelengths. This limitation may affect visualization of particular fluorochromes, which limits the types of assays and quantitation that can be performed. As a result of these problems, LEDs are used increasingly in microscope systems. LEDs can be supplied as either a white-light source, similar to lamp-based illumination, or as fixed wavelength units. In the latter case, units can be interchanged to provide a wide palette of excitation wavelengths if required. One advantage of LEDs over conventional lamps is their long lifetime (approximately 20,000 h), which is significant especially in instruments where large numbers of images are acquired on a regular basis. A second advantage is the

ability to switch on and off "electronically" at high speed, which permits faster imaging. A third advantage is the optical stability of LEDs, which can reduce the changes in illumination of samples over time that occur with lamp-based sources. Finally, LEDs operate at significantly lower temperatures than conventional illumination sources, which reduces problems due to thermal expansion of the system.

Objective lenses

The objective lens is probably the most critical component of any imaging system used for quantitation. When deciding which objective lens and magnification to use for any situation, there is a trade-off between obtaining high resolution images and the required number of optical fields. For screening experiments, where there may be thousands of wells to image, a low magnification lens typically is used that enables the capture of as many cells in as few fields as possible. Capturing images at low magnification, however, can cause problems for quantifying cells. The inevitable crowding as cell densities increase can make the identification of individual cells difficult. Consequently, when imaging at low magnification, careful optimization of cell plating densities is required. For more detailed quantitation of sub-cellular localization of a signal, for example, higher numerical

aperture (NA) lenses usually are required. These lenses access fewer cells/field, but make quantitation somewhat easier.

Another factor to consider when selecting an objective lens is that in general, most automated microscopes/HCIs are limited to the use of air-immersion lenses, which limits the NA that can be used. Manufacturers now are supplying high NA air-immersion lenses of 60 and 100 x. Consequently imaging thousands of fields/conditions at high resolution now is possible using automated microscopy systems.

Detector photomultiplier tubes vs. solid-state cameras

Solid-state cameras tend to be the favored option, because of the requirement for high-speed acquisition of large numbers of images. Solid-state devices can contain cooled charge coupled devices (CCDs), electron multiplying charge coupled devices (EMCCDs) or scientific complementary metal oxide semiconductor (sCMOS) sensors. All of these sensor types offer large dynamic ranges, high frame-rate acquisition, broad spectral sensitivity and high resolution.

Recently, the trend has been toward the use of large format sCMOS sensors for high-sensitivity quantitative imaging to overcome the limitations of EMCCD sensors. While the latter type offers greater than 90% quantum efficiency, its sensor area is relatively small (e.g., 512 x 512 pixels) and its pixel size is large

(typically 16 x 16 μm). Large format sCMOS cameras however, typically operate at > 70% quantum efficiency, but have a much larger sensor area (4–5 megapixels) and a smaller pixel size (approximately 6 x 6 μm). This combination means that for approximately two-thirds the sensitivity of an EMCCD sensor, much larger fields of view at relatively high resolution are available.

Autofocusing

Autofocusing options are among the greatest aids to acquiring large image datasets. Even when using the most expensive plates or coverslips that are manufactured to give the best images possible, there are likely to be minor differences between each focus position from field to field. If uncorrected, these differences would lead to a large number of fields that are out of focus and unusable for analysis. A second major source of focus problems is temperature fluctuation. One can compensate for this by enclosing the optical components in a box, which is how the HCIs typically are constructed.

Software and hardware autofocus systems have been developed to maintain focus over long periods of time. Autofocus systems are available in two versions: software autofocusing, where sequential z-sections are taken and algorithms assess the optimal focal plane; and hardware-based systems. Hardware-based systems usually involve either measuring the z-position of the objective using encoders on

the focus wheel to measure the position of the objective accurately or using laser-based systems that analyze the position of the bottom of a well or slide and monitor deviation from this to maintain focus. The hardware autofocus systems usually account for the fact that the fluorescent signal does not originate from the bottom of the well, but from a little above this. When setting up the focusing for a particular image acquisition, this "offset" is measured on a test field for each wavelength to be imaged. When the system focuses on a particular field, the hardware autofocus finds the base of the well using its laser-based system. The focus then is adjusted by the offset amount and a final very short range software autofocus is used to obtain a final focus position. This enables the system to find focus positions for objects being imaged at multiple wavelengths very quickly and accurately despite changes in the position of the optimal focus and changes in the thickness of the substrate (the plate or coverslip) that the system is imaging through. Software-only autofocusing requires no specialized hardware to implement, but it suffers from the fact that z-sectioning must be performed at each focus position and possibly for each wavelength to imaged. This inevitably slows the imaging process and also can lead to phototoxicity issues.

Confocality

The advantages of confocal imaging for quantitation center about the ability to generate optical slices, where out-of-focus light (thus noise signal) has been removed. Until recently, confocal imaging was limited to conventional microscope setups with all the limitations on throughput that these entail. Automated confocal HCIs are becoming widespread. These instruments use lasers like their conventional cousins, but are more designed for automated imaging of large numbers of fields and wells. Inevitably, confocal optics with up to four lasers means that these instruments are considerably more expensive than their wide field counterparts; however, they enable imaging of three-dimensional (3D) structures and can offer enhanced ability to measure fluorescent signals for both two-dimensional (2D) and 3D specimens.

Software for imaging cytometry

The software of the imaging system is the component with which the user interacts directly; consequently, the software can make collection and quantitation of images either straightforward or complicated. Software falls into two categories: control software for the automated components of the microscope itself and the analysis software.

Control software

For more traditional microscopes with motorized components, the microscope manufacturer produces compatible control software, e.g., Nikon NIS elements (http://www.nikoninstruments.com/en_GB/Products/Software/NIS-Elements-Basic-Research/Brochure) and Olympus cellSens (http://www.olympus-lifescience.com/en/software/cellsens/). An alternative to commercial software supplied with a particular instrument is to use third party software designed to control the various automated components from different manufacturers. Examples include Metamorph (http://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software) and Volocity (http://www.perkinelmer.co.uk/pages/020/cellularimaging/products/volocity.xhtml). An option that increasingly appears in the literature is μManager, an open-source microscope control and acquisition software package (https://micro-manager.org/wiki/Micro-Manager%20Project%20Overview) (Edelstein 2014). Micromanager uses ImageJ (http://fiji.sc/ImageJ) as its basis and can control nearly any microscope-camera combination.

Image analysis software

Both commercial and open-source options are available for image analysis. ImageJ and the closely related package, FIJI (http://fiji.sc/Fiji), provide extensive plug-ins

for image analysis. This type of software operates like traditional graphics packages, however, such as Photoshop, and thus is not ideal for analyzing multiple fields, wells and plates, which typically would be the case for image-based biological screens of small molecules for example.

HCI manufacturers supply instrument-specific software, e.g., Molecular Devices MetaExpress. Where images must be analyzed using different microscope systems, third-party machine-independent software packages are available such as Definiens Tissue Studio (http://www.definiens.com/research), Molecular Devices Metamorph and ImagePro (http://www.mediacy.com/index.aspx?page=IP_Premier). These packages can import images and metadata from different imagers and provide extensive analysis options.

A noncommercial, free, machine-independent option is Cellprofiler (http://www.cellprofiler.org/). Cellprofiler operates similar to commercial high-content imaging software provided with HCIs. Cellprofiler takes images and pushes them through analysis pipelines that are created by stringing together different algorithms to manipulate and analyze images in much the same way as the instrument-specific packages.

Data analysis software

Once images (image stacks) have been analyzed, a way is needed to visualize and assess the data statistically. Traditional statistics software suites such as SPSS and Graphpad can be used, but these were not designed for the types of analysis considered here. Commercial suppliers of HCIs produce software packages that can visualize the analyzed data and the linked images from an image stack or multiple stacks, i.e., Acuity Xpress from Molecular Devices (http://www.moleculardevices.com/systems/high-content-imaging/acuityxpress-high-content-informatics-software). An alternative to commercial offerings are open-source packages. Cellprofiler analyst (http://www.cellprofiler.org/) is a free package that was designed to analyze multidimensional high-content data and it includes the capability for machine learning of phenotypes. Bioconductor (https://www.bioconductor.org/), which is based on the statistical package R, contains numerous packages for analyzing a wide variety of biological data, including microscopy-based data (e.g., https://www.bioconductor.org/packages/release/bioc/html/imageHTS.html).

Types of information extractable from images

The information that can be extracted and analyzed from images is limited only by the probes used and the ability to obtain good images. I discuss some major categories briefly below.

Morphology

Morphological analysis of bright field (typically phase contrast) images is extremely challenging. Although some algorithms have been developed (Cordelieres 2013), morphological analysis based solely on bright field imaging is not used routinely. Morphology visualized by fluorescent probes can be used routinely to measure features. Complications can occur when multiple structures lie close together, e.g., using dyes and probes that mark the cell's edge or fill the cytosol. Many dyes can be used for such purposes; for details see https://www.thermofisher.com/uk/en/home/references/molecular-probes-the-handbook/fluorescent-tracers-of-cell-morphology-and-fluid-flow/choosing-a-tracer.html. An alternative to the use of dyes is the use of fluorescent proteins such as eGFP (Shaner 2005). These may be either constitutively expressed in their native form or linked to tags to direct them to the plasma membrane or nucleus for example (Shaner 2005). Probes that demonstrate a cell's shape, however, may not always give clear demarcation between adjacent cells, particularly with a high local density of cells. Consequently, sophisticated algorithms may be needed to find the boundary between adjacent objects. Neuron morphology presents a challenging problem for quantitation, because the structures are complex and often overlap in 3D, which confounds the analysis. Several methods now exist to address these

problems and can be used to measure features of neurons (Meijering 2010). Nevertheless, tracing neuron morphology remains a relatively difficult problem.

Intensity of fluorescence

By far the most common features extracted from images are related to the fluorescence intensity in individual cells. These types of analysis critically depend on having staining controls that allow the investigator to distinguish objectively signals from background. Also valuable is a positive control that allows the investigator to assess whether, with the acquisition and analysis settings used, the desired signal can be seen and, more importantly, if signals that are weaker, but still above background, can be identified. At the simplest level, a negative control for a stain makes counting the cells that are positive for the signal straightforward. Owing to the single cell nature of the analysis, distributions of different fluorescent signals can be obtained that are useful for assessing factors such as heterogeneity of samples. Once measurements have been obtained, it is relatively simple to count the number of cells that are positive for a particular fluorescent signal or to obtain frequency distributions or higher dimensional analyses. There are many examples of such applications of fluorescence intensity including cell proliferation estimates based on cell counts, degree of antigen expression (Gasparri 2005), the occurrence

of apoptosis (Gasparri 2005) and expression of surface markers that indicate the state of pluripotent stem cells (Barbaric 2011).

Distribution of fluorescence

One of the most useful features of imaging cytometry is its ability to analyze the spatial distribution of a signal within cells and under different experimental conditions. Two general methods are used to achieve this. The first uses reagents to mark different parts of a cell, which allows these parts to be identified and linked together to form a virtual cell. The fluorescent signal of interest now can be measured in each identified part of the cell and any translocation will cause an intensity change in different regions of the cell. For example, a nuclear counterstain, such as DAPI, can be used to locate each cell unambiguously. Next, a second stain, e.g., CellMask stains (https://www.thermofisher.com/uk/en/home/references/molecular-probes-the-handbook/probes-for-organelles/nuclear-and-chromosome-counterstaining-and-nissl-stains.html), can be used to locate the cytosol. The boundary of each cell can be extracted from the images to identify the cytosol area. The fluorescent signal of interest then can be measured in the nucleus and cytosol. The analysis of fluorescence distribution is particularly relevant for studying signaling during which intracellular molecules translocate (Caunt 2008).

The second method relies on the fact that the cytosol surrounds the nucleus. The nucleus is located within the image (see below) and a mask is created that tells the software which pixels correspond to the nucleus. This nuclear mask can be copied and expanded so the outer portion lies over the surrounding cytosol. The nuclear mask can be subtracted from the expanded mask to leave a ring-shaped mask that no longer contains the portion of the mask over the nucleus. The ring mask can be applied to the fluorescent channel whose distribution is of interest, which provides a value for the fluorescence signal in the cytosol. The corresponding nuclear mask also can be applied to the same image to obtain a value for the signal in the nucleus; therefore, the ratio between the fluorescent signals in each compartment can be assessed.

Cell behaviour over time

An increasingly important method for investigating cells is by time-lapse analysis and cell tracking. Cells can be grown in environmental chambers within microscopes that can maintain temperature and partial pressure of CO_2. By tracking individual cells, detailed information can be obtained about cell behavior that would not be seen using conventional sampling strategies. For example, human embryonic cells in culture do not readily re-form cultures from single cells (Enver 2005). Detailed tracking revealed that there were several impediments that

the cells had to overcome during re-growth experiments (Barbaric 2014). Identification of these impediments was made possible by imaging the cells during several divisions and analyzing the resulting images. This also emphasizes that one of the challenges of using image analysis is to keep track of each cell.

Cell tracking can be particularly difficult when dealing with cells growing at high density and where there is considerable cell movement, whether natural or induced by experimental conditions. Fluorescent labeling of cells can help identify and track them owing to the relative ease by which fluorescent signals can be detected in images. Strategies for labeling cells range from the use of "cell tracking dyes" to genetically labeling cells with fluorescent proteins, such histone-H2B-GFP, which labels nuclei (Kanda et al. 1998). Label-free imaging is far more challenging owing to the nature of bright field images such as those associated with phase contrast optics, where, unlike fluorescent images, the pixels that make up the background of the image are close in greyscale values to actual the pixels that denote the object to be extracted from the image. **S**oftware packages now are available, however, that can track and quantify cells (Piccinini 2015).

Higher order structures

Objects contained within images such as nuclei, cells etc are often part of bigger objects within the image and it is often desirable to link objects to these higher-

order structures. A simple example is a colony of mammalian cells growing in vitro, where a researcher may want to know the number of cells (nuclei), the number of colonies and how many cells are within each colony. Once the pixels associated with each individual cell has been isolated from an image and measurements of features made, the collection of cells can be associated with the colony from which they came. The colony has features that are associated not only with itself, e.g., area and perimeter, but also features that are measured from the cells within the colony; therefore, measurements such as the number of cells/colony and how many are positive for a certain marker protein, can be obtained (Barbaric 2011). Another example of higher-order structures linked to single cell information follows from the recent development of tools that allow zebrafish embryos to be imaged and analyzed quantitatively in high-throughput drug discovery investigations (Peravali 2011).

How is quantitative information extracted from images?

The basic aim of any quantitation is to extract the desired signal from images and to discard unwanted signals. The availability of high-quality images can be critical to ensuring smooth analysis. This process can be divided into the following steps: image acquisition, image pre-processing, **separation of the desired objects pixels**

from the rest of the image (segmentation), feature extraction and classification (Fig. 1).

Image acquisition

Critical factors for obtaining useful images for quantitative analysis are the same as for standard microscopy, except that additional consideration may be required for the automated nature of the process. The fluorescent probes used, e.g., dyes or antibodies, should provide good signal-to-noise ratio staining and permit well-focused images. When establishing acquisition parameters, care must be taken to balance acquisition time against the intensity of signal to ensure that enough of the camera's dynamic range is used to allow signals of different intensities to be imaged using the same exposure time.

Consideration also must be given to the balance between sufficient resolution for the structures to be imaged and imaging a sufficient number of cells to obtain statistically relevant data. The ability to image features correctly depends, in part, on the substrate through which the cells are observed. Conventional tissue culture plates, such as those with 6-, 12-, 24- or 48- wells, typically have bases that are 800−1200 μm thick and may not be perfectly flat; this reduces signal strength. Specialist plates, which typically have ≥ 96 wells, possess much thinner bases (approximately 170 μm), which increases signal strength and aids focusing.

For accurate quantitation it may be necessary to perform a background correction. One type of background problem is uneven illumination caused either by the light source or an uneven plate. Arc-based illumination systems may give weaker illumination at the edges of wells; however, this effect can be reduced by using of non-arc based sources such as LEDs. Where detailed quantitation is required, corrections can be made to the images prior to analysis. These corrections involve imaging an empty well to calculate a "flat field." This correction must be applied to each wavelength used to instruct the software how to correct the background illumination to provide uniformity across the field of view. An additional source of uneven illumination may result from the plates that have been manufactured with an increased base thickness near the well edge. This usually can be mitigated by avoiding imaging close to the edge of a well. A summary of some of the factors that should be considered is provided in Table 1

Segmentation

Segmentation is the automated process of extracting objects from images. Certain properties of these objects then are measured. Each segmentation of a particular set of objects generates a mask, which tells the software which parts of the original image to include and which to ignore. This mask is applied to the original image to generate a separate, processed image in which only the desired pixels are used for analysis. The original image is not altered and the downstream processing and analysis uses the original image data. The simplest type of segmentation is simply

to set an intensity threshold; below a certain threshold of grey levels in the image the pixels are ignored by the image processing algorithms in any subsequent analysis steps. This has the advantage of relative simplicity, but can be affected by image illumination defects. More commonly, tailored segmentation algorithms are used to extract features from particular cellular objects, e.g., neurites or nuclei. Such algorithms can account for particular features of an object, e.g., the range of sizes of a vesicle, and in this way achieve more accurate segmentation.

Feature extraction and classification

Once images have been segmented and objects have been extracted from the images, a wide variety of measurements can be applied to each. For example, the measurement of the average fluorescent intensity across an object, or the total integrated fluorescent signal contained within an object, would allow frequency histograms or scatter plots to be constructed from single cell data. Morphometric measurements can be as simple as the area or perimeter of each object. More sophisticated morphometric measurements, however, also can be applied. Examples include the perimeter of an object, which can include the internal perimeter of "holes" within an object, maximum distances along the x and y-axes of an object, or chord lengths within an object. Most analysis software can measure properties of fiber-like objects, e.g., neurites. Such properties include how many end points a fibrous object has, how many bifurcations there are, or even how many times a fibrous object is crossed by another object. Because they are

determined and reported at the level of each individual object, all such measurements can be associated with an x,y,z position; therefore, the spatial relation between objects can be established.

Future directions

The current state of HCI technology enables relatively sophisticated analyses to be performed on large numbers of cells. An obvious question is how the capabilities of high-content analysis will evolve in the future. One area where improvement has begun, and further progress seems likely, is acquisition speed. The use of solid-state devices for illumination together with sensitive, large-format sCMOS cameras, means that acquisition times/field of view and the number of fields that can be photographed/hour, are increasing. Another area where further development seems certain is the use of confocality in imaging systems to enable accurate quantification of multi-layered objects and image smaller (sub-cellular) objects. Associated with the introduction of confocal optics is the use of high magnification air lenses that can take advantage of the high resolution imaging capabilities afforded by confocal optics to enable the investigation of subcellular objects in large numbers of cells and conditions. Another fascinating development is the introduction of super-resolution technologies for standard research microscopes. This should allow unprecedented quantitative imaging of subcellular structures

previously only available by using electron microscopy (Jones et al. 2011). It is likely that even with the current level of technology, HCI analysis will be used increasingly for basic biomedical research. The increased availability of automated imaging systems, sophisticated software for analysis and fluorescent probes means that quantitative analysis of microscopy images is likely to become more routine in research.

Declaration of interest: The author reports no conflicts of interest. The author alone is responsible for the content and writing of this paper.

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Table 1:Factors to be considered in the experimental setup to enable extraction of quantitative data

	Factors to consider	Notes
Imaging set-up		
	What features need to be analyzed? Are suitable probes available?	
Resolution	What resolution is required?	Subcellular structure would typically need 20 x. High NA lenses are more difficult to use on tissue culture plates (may need to use slides).
Colors	Can you image enough colors?	Maximum typically = 4 (Sedat quad sets). Reporters may need specific filter sets.
Plates	Can you see enough biology in one well? Is the signal strong enough to image in a reasonable time?	A 10 x lens requires ~ 100 fields to cover one well of a 24-well tissue culture plate. 6-well plates have bases ~800−1200 μm thick, which reduce signal strength. Consider specialist imaging plates.
Image analysis		
Segmentation and feature extraction	Are there suitable controls?	Ideally, there should be positive and negative controls to ensure that the biological variation can be captured.
	Can you segment objects accurately?	Consider using a higher magnification.
	Have enough object been imaged?	Image more fields /well or more wells.

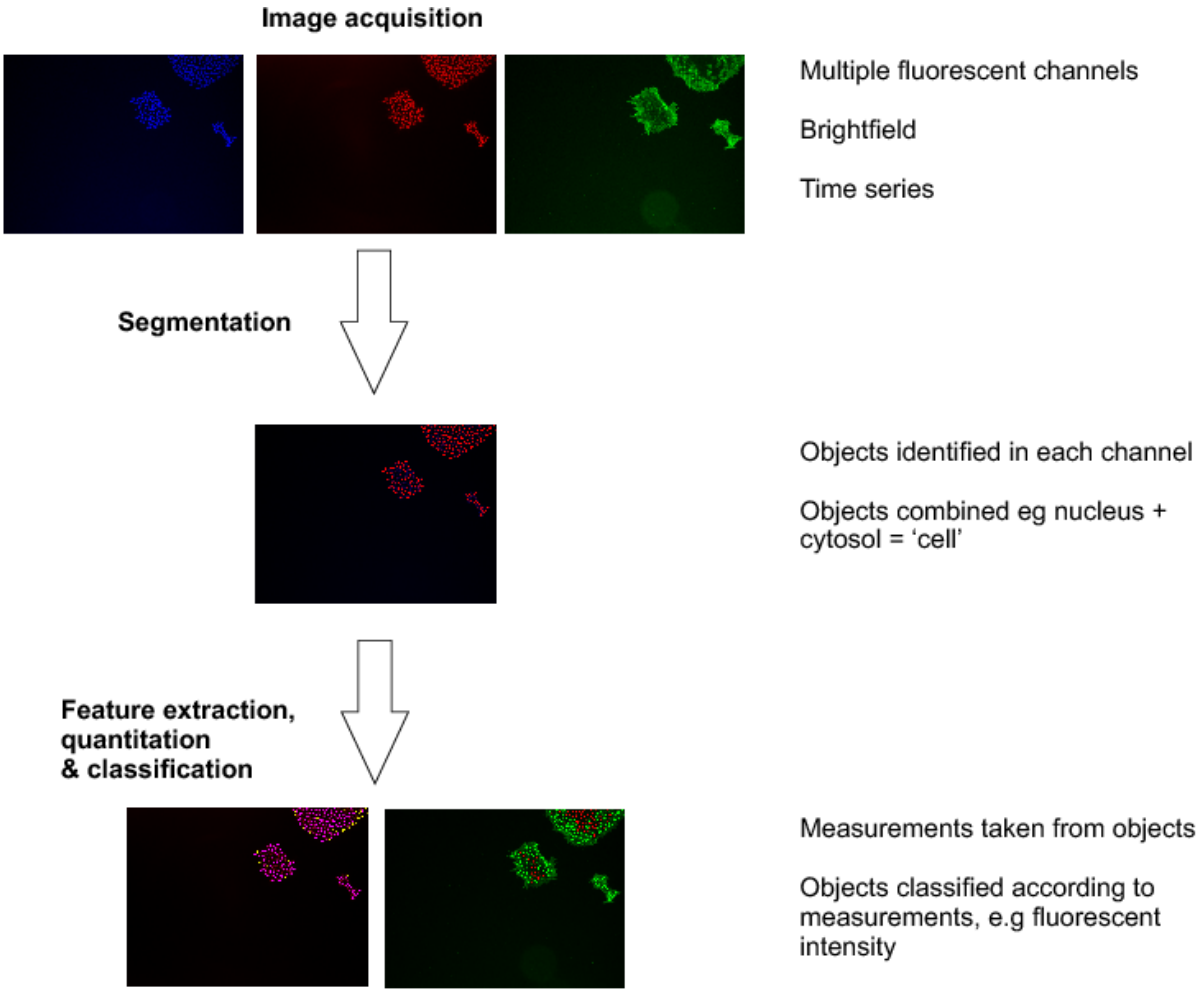

Figure 1: A summery of the process of extracting quantitative inofmation from microscopy images. Image acquisition can involve multiple wavelengths (channels) including bright-field and time series. Objects are extracted from images using specialized segmentation algorithms that are optimised to extract objects within images that may have different morphological and fluorescent properties. Segmented objects can be combined to link features for example in the same cell. Measurements are then taken from each segmented object on a cell by cell basis and can then be analysed at the single cell or as an average of many cells in a particular experimental condition.