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Characteristics and Value of Directed Algorithms in High Content Screening

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Summary

High content screening requires image processing algorithms that can accurately and robustly analyze large image numbers without requiring human intervention. Thus, a suite of algorithms that are directed by an understanding of the biology being studied was developed for the optimized automated acquisition and quantitation of cellular images. Two categories of directed algorithms were developed: Developer Tools for assay development and Specific Algorithms for turnkey screening of specific biological situations. The same basic sequence of analysis steps are used in these directed algorithms:

1. Primary object identification.
2. Measurement of primary object properties.
3. Identification and measurements of associated targets.
4. Analysis of raw measurements for specific biological problems.

The detailed application of these steps is guided by the biology being studied and the expected phenotypic changes. Most cell biological problems to be analyzed using high content screening can be categorized by either the phenotype of the problem or labeling pattern, or by a standard biological response behavior of the cells. This enables application of directed algorithms optimized for these categories. Examples of the use of directed algorithms for specific categories are discussed, as well as the detailed analysis steps for a specific directed algorithm.

Key Words: Directed algorithms; HCS; high content analysis; high content screening; image analysis; quantitative fluorescence imaging; translocation.

1. Introduction

Quantitative fluorescence microscopy, in which the images of the fluorescently labeled biological samples are quantitatively analyzed, takes microscopy from being a purely visual, descriptive, and subjective tool to one of much more power by offering an objective, quantitative dimension. Quantitative analysis of the cellular fluorescence can provide information on the spatial distribution, amounts and arrangements of the fluorescently labeled macromolecular targets, and the morphology of the cells. Fluorescence enables multiple fluorophores with different emission wavelengths in the visible spectrum to be used to simultaneously label and quantitatively monitor and correlate multiple targets. Cells can have a heterogeneous response; quantitation enables subpopulations of cells to be identified that exhibit the different categories of responses, leading to identification of more nuanced responses to changing biological conditions. The advantages of quantitative fluorescence microscopy in studying cells are further enhanced by the

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addition of automation. High content screening (HCS), or the automated acquisition of the fluorescently labeled cellular images and their subsequent automated analysis enables the quantitative assessment of large sample numbers leading the way for large scale biology. This automated approach requires image-processing algorithms that can accurately and robustly analyze large image numbers without the need for human intervention.

Over the past 20 yr, a growing number of image processing software packages have become available enabling users to write image analysis programs using core image processing functions (e.g., MetaMorph, ImagePro, and Axiovision). Each package allowed users to interactively determine a sequence of image processing steps to analyze their images, and then recorded these steps so that they could be reused for analysis. However, these recorded scripts could be used only for a limited set of similarly acquired images and biology. Such image processing and analysis approaches were not sufficient to meet the needs of analyzing large numbers of images or images acquired under differing imaging conditions in an accurate and robust manner.

The same key basic sequential analysis steps can be applied to most cell biological problems requiring the analysis of microscope images of fluorescently labeled cells (described later in this chapter). However, the specifics on how these steps are to be applied require an understanding of the biology that is being analyzed, and the expected phenotypic changes. Some of the changes that could be expected are changes in cell shape, movement of the cells, rearrangement of intracellular targets of interest, or an accumulation of particular macromolecules. We call this biological understanding of the problem being studied and the resulting informed expectation of the phenotypic changes as the "domain knowledge" of the biological system. Domain knowledge enabled us to develop directed image processing algorithms that could be optimized and tuned for specific types of biology, and report quantitative features appropriate for the biology. In other words, two options exist for analyzing a fluorescence microscopic image of cells: (1) the undirected approach in which domain knowledge is not needed, and everything in the image that is possible to measure is measured, and the measured features, which change between the positive and negative control cases are monitored and (2) the directed approach in which the domain knowledge is used to measure the properties of the cells in the image which are relevant for the biology, and also used to understand the constraints on the biological problem to facilitate and simplify the image processing. For example, an image-processing algorithm for the quantitation of neurite outgrowth would be guided by the domain knowledge of the morphology and arrangements of neurons and neurites, and would be optimized for this class of objects. Properties measured that are relevant for this type of biology include the number of neurites per neuron and the neurite length. A very different algorithm would be needed to quantify the translocation of a protein from the cytoplasm to the nucleus; in this case, domain knowledge would guide the algorithm to identify the nuclear and cytoplasmic regions of the cells, and then measure the intensity in these distinct cellular regions, as well as calculating the intensity ratios and differences between these regions as optimized metrics to quantify the translocation event.

Our strategy for developing image-processing software is to develop a suite of algorithmic software modules, also known as BioApplications, which carry out the automated acquisition and quantitation of cellular images in an optimized manner that is directed and guided by the domain knowledge of the biology being studied. Within the domain of particular biological situations, the relevant algorithm makes a wide range of measurements relevant to the problem being studied, and the algorithm needs only minor adjustments to deal with the particular biological situations it was being applied to or to images acquired under differing imaging conditions. For example, one algorithm is designed to simultaneously quantify and correlate the translocation of several proteins from the cytoplasm to the nucleus; to run this algorithm, the user only needs to adjust settings for intensity thresholds and object identification criteria to take into account the particular cell type, intensity staining level and imaging conditions under which the experiment was done. The basic sequence of steps identifying the nucleus, cytoplasm, the translocation of the different

fluorescently labeled targets between these distinct cellular regions, and the correlation between the different targets analyzed, are all done automatically by the algorithm, without the need for the user to redefine the image processing steps each time the software is used.

In the next few sections of this chapter, we describe the strategy and thinking employed in developing these directed image-processing algorithms. We first describe the requirements needed for directed algorithms and the general analysis steps that are universal for HCS problems. We then describe categories of HCS problems defined by their biology or phenotypic response, and how this domain knowledge led to directed algorithms for their analysis. We end the chapter describing in more detail the specific analysis steps in one of our directed algorithms.

2. Categories of Directed Algorithms for HCS Assays: Developer Tools vs Specific Algorithms

While developing a comprehensive suite of directed algorithms, we found two distinct sets of capabilities that were required by users from their image processing algorithms. The first category of use and requirements was that the image-processing algorithms needed to be fairly general applications that could be easily configured and applied to a wide range of cell biological situations. These applications provided more of basic information about individual cells and cell populations, and were fairly flexible in their usage and application. Users having these requirements were typically involved in doing basic cell biological research, or were in drug discovery and were doing target identification, target validation, or screen development; all areas that required a flexible tool that could be easily configured and applied to give more of basic quantitative information about the state of cells. These users frequently had a sophisticated understanding of HCS technology, as well as its applications and potential, and required a tool that would allow them to develop their own approaches. The other category of use and requirements was that the image processing algorithms needed to be simple to use with rapid start-up time. Turnkey usage was the emphasis, such that a person with less sophisticated understanding of the details of HCS technology could still easily conduct HCS assays. In this case, the image analysis was targeted to very specific biological situations with their specific set of measurements, and any interpretive logic or additional analysis of the raw measurements pertinent to the biological situation was built in, adding to the application's "turnkey" design. Users in this category were often involved in primary screening efforts.

In our suite of directed image processing algorithms, we developed two categories of algorithms fitting the above sets of requirements: Developer Tools and Specific Algorithms. Developer Tools were designed as general purpose assay development tools, whereas Specific Algorithms were designed to be a turnkey solution. The user developing a HCS assay using the functionality offered by a Developer Tool can develop, customize, and validate the assay for their particular biological situation. Developer Tools have a broad biological range in which they can be configured and applied to a wide variety of cell biological situations. These algorithms make and report a large set of basic cellular domain measurements with their statistics, thus giving these tools a high degree of flexibility. Developer Tools were not designed to provide additional interpretive logic or analysis of the raw cellular measurements for particular cell biological situations; for these the Specific Algorithms are used.

Specific Algorithms were designed to be easily and rapidly applied for the screening of targets of a specific biology. They were designed to have rapid startup time, validated protocols, and specific assay classification features, all targeted at a specific biology with a specific set of assay measurements. There is flexibility, but this is within the targeted biological problem being solved. Classification of categories specific to the targeted biology are automatically provided, and logic to interpret results is integrated into the analysis.

Developer Tools are still directed algorithms in that they are still guided by the general biological context in which the measurements are made. However, domain knowledge is required

for users to set the input parameters to focus these tools toward specifically analyzing a particular biology. In other words, both Developer Tools and Specific Algorithms are directed algorithms, with the Specific Algorithms being very biology driven and deeply analyzing its specific biology, whereas the Developer Tools can embrace many more biologies, but with a more shallow level of analysis. For example, a Developer Tool like Cellomics' Compartmental Analysis BioApplication is designed to identify several distinct regions within a cell, and then report properties related to the fluorescent target's intensities (e.g., differences, ratios) within these intracellular regions or compartments. Thus, domain knowledge directs the algorithm to identify the cell, the intracellular regions, and then make the measurements in the identified regions. In contrast, a Specific Algorithm like Cellomics' Cytoplasm to Nucleus Translocation BioApplication only identifies two intracellular regions, the cytoplasm and the nucleus, and makes intensity related measurements between these two regions. This too is a directed algorithm, but with a narrower scope, than the more broadly applicable Developer Tool.

3. Analysis Steps in Automated Directed Algorithms

In directed algorithms for HCS, whether a Developer Tool or Specific Algorithm, the same basic sequence of key analysis steps are employed. However, before applying any type of analysis, the user must identify the biological question he/she is trying to answer. Being able to articulate the question being answered enables deciding the objective of the analysis. Once this critical, but often overlooked step has been done, then the directed image analysis strategy can be devised. The steps and their sequence in the automated directed analysis approach are as follows:

Fig. 1. *(Opposite page)* Applications of directed algorithms to four different biological situations. The top row shows the raw fluorescence image, and the bottom row shows the overlays applied by the directed algorithm on automatically identifying key entities to analyze. First Column: Images of NF-κB translocation from the cytoplasm to the nucleus analyzed by Cellomics' Molecular Translocation BioApplication; specifically shown are the target channel (nuclear factor [NF-κB] labeling) in which the cells were stimulated with IL-1α to induce translocation from the cytoplasm to the nucleus. Note that in some cells translocation has not occurred, and the NF-κB is mainly in the cytoplasm. The color overlays show results of analysis by the Molecular Translocation BioApplication. The primary objects are the nuclei, which fit the criteria to be selected for analysis; these criteria were set such that binucleated cells were not analyzed, and thus the rejected objects have no overlay. The nuclear region is shown by a red overlay. The annular part of the cytoplasm that corresponds to the cytoplasm, and from where the cytoplasmic intensity was measured, is shown by a green overlay. Second Column: Image from the analysis of F-actin and whole cell morphology by Cellomics' Morphology Explorer BioApplication. The raw image shows the F-actin target, and the color overlays show the whole cell (yellow) and the F-actin fibers (red) that were identified and analyzed by the Morphology Explorer BioApplication. The primary object, the whole cell, was identified by a whole cell stain (not shown). Several of the cells were touching so advanced segmentation was used to separate and resolve the individual cells (yellow overlays). Third Column: Analysis of embryonic stem cell colonies grown on a feeder layer of mouse fibroblasts by Cellomics' Compartmental Analysis BioApplication. The raw image shows the cell nuclei labeled with Hoechst 33342. The primary object is the stem cell colony, but the image also has the smaller nuclei from the feeder layer cells. The overlays show identification and analysis by the Compartmental Analysis BioApplication. A size exclusion criteria was set to identify the large object as the primary object (stem cell colony) and retain it for analysis (blue overlay). Smaller objects were rejected from analysis (orange overlay), as they corresponded to the feeder layer cells' nuclei. Once the primary object was identified, the oct4 labeled nuclei in the target channel could be identified and counted, to calculate what percentage of the stem cells in the colony were pluripotent (not shown). Fourth Column: Analysis of angiogenic tube formation by Cellomics' Tube Formation BioApplication. The raw image of the connected angiogenic tube (primary object) is shown. The overlays show the results of the analysis by the Tube Formation BioApplication. The algorithm identifies the connected tube (primary object; blue overlay), and branch nodes (pink dots). Unconnected tube segments are also identified and shown by a light blue overlay, and debris rejected from analysis are shown by an orange overlay.

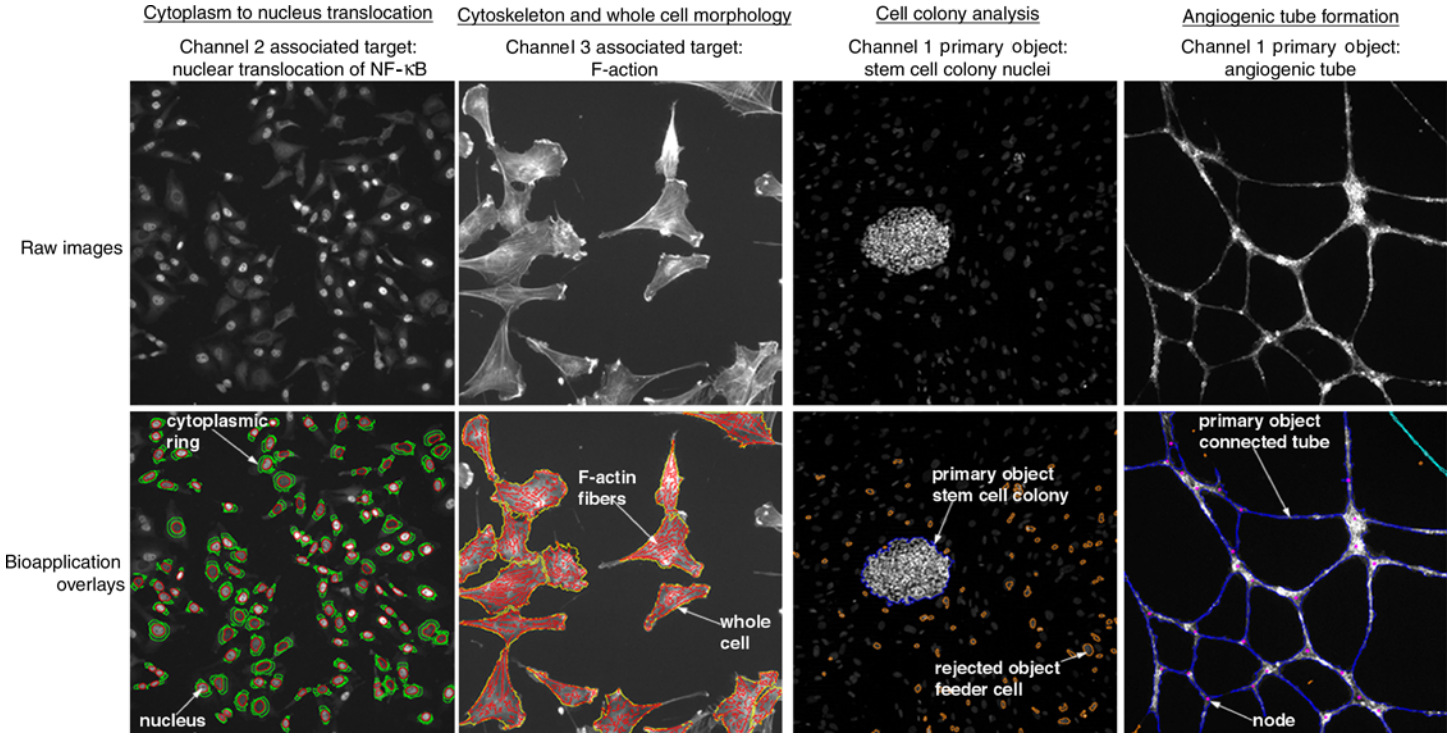
Cytoplasm to nucleus translocation
Channel 2 associated target:
nuclear translocation of NF-κB
Cytoskeleton and whole cell morphology
Channel 3 associated target:
F-action
Cell colony analysis
Channel 1 primary object:
stem cell colony nuclei
Angiogenic tube formation
Channel 1 primary object:
angiogenic tube
Raw images
Bioapplication overlays
cytoplasmic ring
nucleus
F-actin fibers
whole cell
primary object stem cell colony
rejected object feeder cell
primary object connected tube
node

Fig. 1.

Fig. 2. Fluorescence intensity distribution within a cell at different stages of the transcription factor activation process: **(A)** before activation, **(B)** during activation, **(C)** after activation.

3.1. Accurate Identification of Primary Objects

Directed high content analysis starts with the definition and identification of a primary object. The primary object defines:

- The main object being analyzed, and thus also enables its counting.
- A region(s) in which other objects or targets, associated with the primary object, are analyzed.

Examples of primary objects include the cell's nucleus, other cellular organelles such as the Golgi, individual cells, multicellular assemblages such as colonies of tubes, tracks made by cells, and holes in objects (**Fig. 1**). An understanding of the biology being studied guides the choice of the appropriate primary object for the HCS assay. Examples of associated targets could include the cell cytoplasm or other intracellular targets such as organelles and cytoskeleton (primary object is either the nucleus or the whole cell), individual cell nuclei in a colony (primary object is the cell colony) or even individual cells when the primary object is based on an area of the extracellular space (**Fig. 1**).

Initial identification of the primary object is usually made based on segmentation and intensity thresholding, followed by application of additional intensity, connectivity, and morphology criteria to select the correct objects for analysis. Domain knowledge of the biological problem being solved is critical to the accurate identification and analysis of the primary object; it is required for appropriate segmentation, definition of the region of interest for analysis, and application of correct object rejection and selection criteria relevant to the biology being studied. Ignorance of the biological context of the problem might lead to misidentification of the primary objects to analyze, leading to irrelevant results. **Figure 1** has examples of different primary objects identified for different biological situations.

3.2. Measurements of Primary Object Properties

Once the primary objects have been correctly identified, raw measurements are made on them. The raw measurements could include shape and size measurements, measurements of the label's intensity and texture, measurements on any extensions from the primary object such as cellular processes (e.g., neurites from a neuron), and even measurements on the proximity or spacing of other objects. Domain knowledge directs that measurements appropriate to the biology being studied are made. For example, if the primary object was an angiogenic tube, then appropriate measurements relevant to the biology would include the tube's length, and the number of branch nodes (**Fig. 1**). These measurements do not have relevance in a situation in which the primary object is a nucleus and the biological problem being studied is the intracellular translocation of a protein (**Figs. 2–6**); in this case the nuclear shape, size, and intensity might be the relevant measured features, so that the cell could simultaneously be monitored for toxicity (seen as nuclear condensation or fragmentation) or changes in cell cycle state (changes in nuclear intensity).

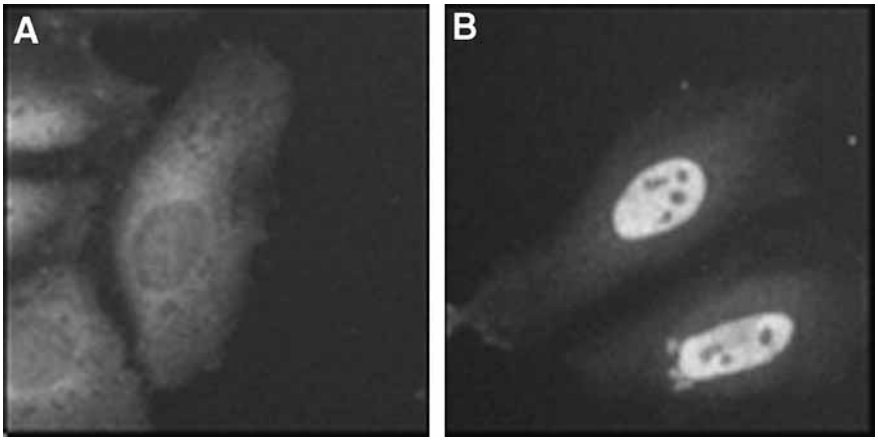

Fig. 3. Translocation of NF-κB from the cytoplasm to nucleus: **(A)** before activation and **(B)** after activation.

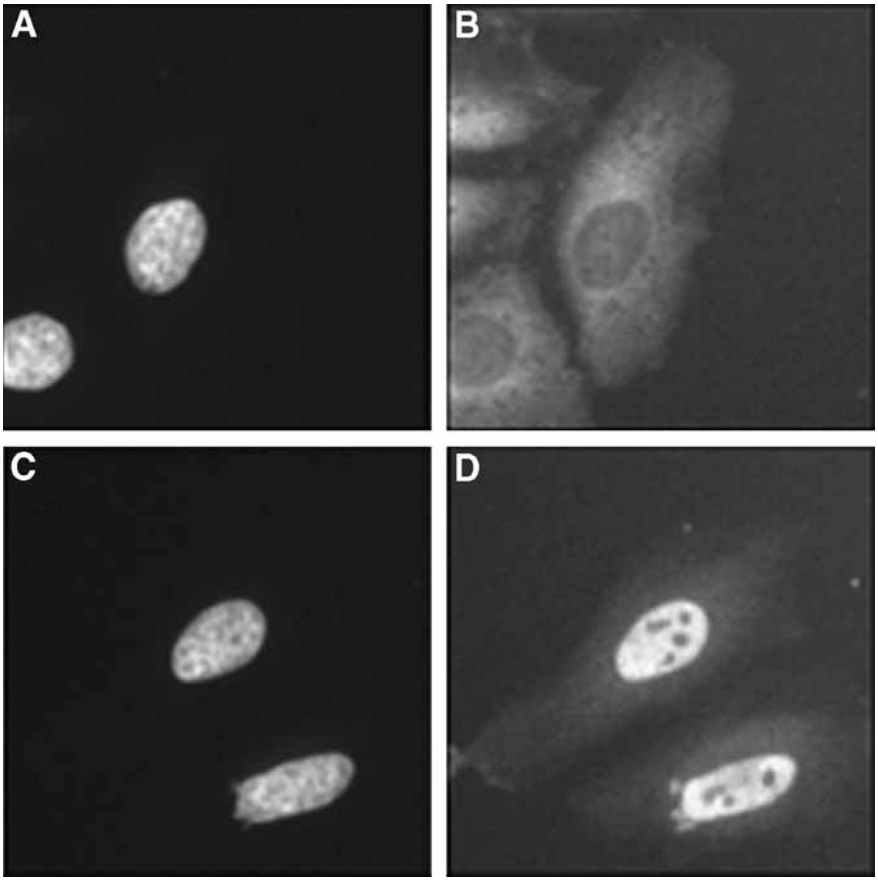

Fig. 4. Two color fluorescence labeling. Before activation: **(A)** nucleus channel; **(B)** target channel. After activation: **(C)** nucleus channel; and **(D)** target channel.

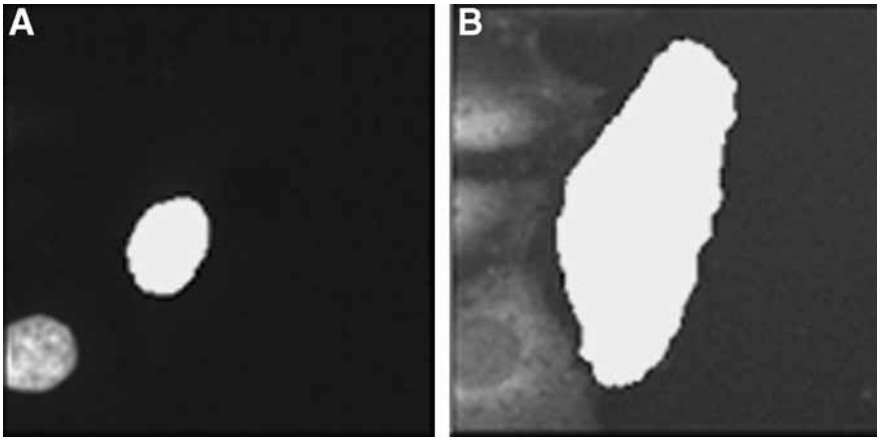

Fig. 5. Results of an object (nucleus) and target (cytoplasm) identification shown as white overlays on gray scale images. **(A)** Nucleus mask and **(B)** cytoplasm mask.

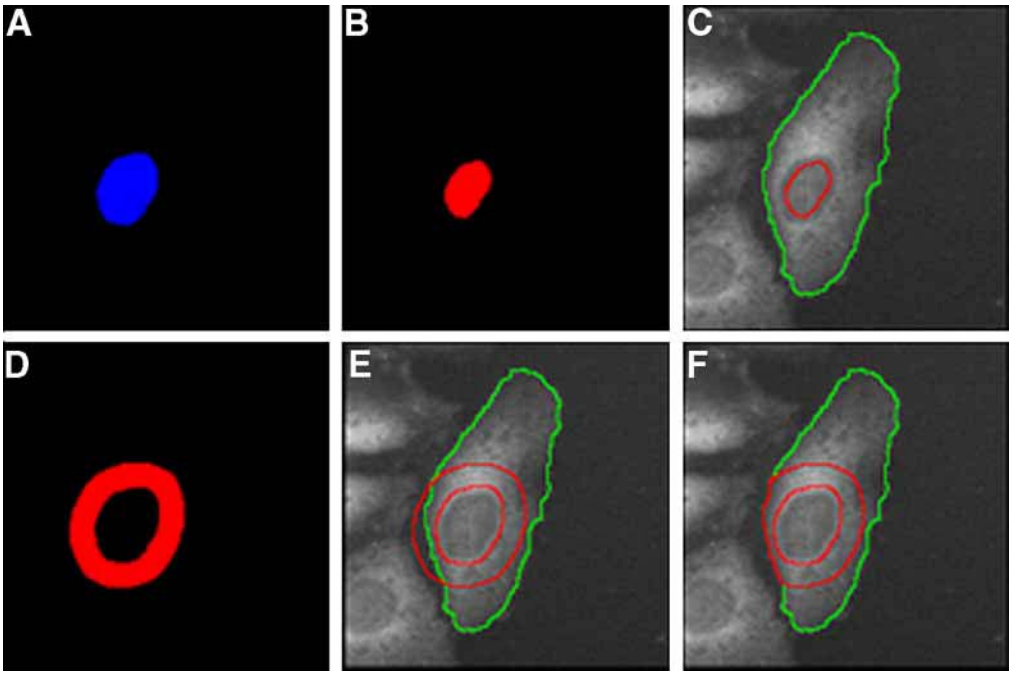

Fig. 6. Masks used to extract measurements: **(A)** original nucleus mask; **(B)** CIRC mask, erosion applied; **(C)** outline of CIRC mask projected on the cell image from the target channel; **(D)** RING mask with inner distance = 1 pixel and width = 12 pixels; **(E)** outline of RING mask projected on the cell image from the target channel; **(F)** outline of the target confined RING mask projected on the cell image from the target channel.

3.3. Identification and Measurements of Associated Targets

The next step is to make raw measurements of entities associated with the primary object, but labeled with other fluorophores. This is the basis of HCS' multiplexing capability, and its "high content" information. Again, understanding of the biological problem is critical in guiding the algorithm to correctly identify the entities to be measured, and then to make the appropriate

types of measurements. For example, with the Morphology Explorer BioApplication one can use the whole cell as the primary object, and then can identify fluorescently labeled cytoskeletal fibers (e.g., F-actin or microtubules) inside the cell (**Fig. 1**). Based on an understanding of typical cytoskeletal shape, the BioApplication can specifically identify long fiber-like structures inside the cell amidst background fluorescence noise. Once the fibers are identified, it reports relevant properties of the fibers for each cell, such as the number of fibers, their area, and various metrics related to their intracellular alignment.

3.4. Analysis of Raw Measurements Tailored for Specific Biological Problems

Once the raw measurements have been made, additional logic and analysis of raw measurements specific to biology being studied can then also be applied. This additional analysis, which results in answers relevant to the biology being studied comes from an understanding of the biological context of the assay and problem. This additional analysis is a critical aspect of directed algorithms. It is characteristic of Specific Algorithms, but Developer Tools also offer additional analysis, which results in relevant measurements for particular biological situations.

Ultimately, the questions being asked of the cells in HCS assays are whether they are responders for a particular situation, and the degree of their response. A knowledge of the specific biological situation enables proper interpretation and reporting of the response. Thus, the goal of the additional analysis of the raw cellular measurements is to come up with quantitative features that both sensitively and robustly report the cellular response.

Some of the additional analysis are simple arithmetic functions of the raw measurements, which provide a more sensitive quantitative metric for the biological process being studied. For example, after identifying and measuring the intensity in the cytoplasmic and nuclear regions of the cells, several algorithms will then also report the difference and ratios of these intensities as more sensitive measures for the translocation of any protein between these two distinct cellular regions. Often, "indices" appropriate for the particular biology being studied might be defined, and characterize the overall response in the image or the well. For example, in Cellomics' Tube Formation BioApplication, an "Angiogenic Index" is calculated, which is proportional to the percentage of an image's area that is covered by a connected angiogenic tube; this simple metric indicates the level of angiogenic tube formation within the well. Another example is the "Neurite Outgrowth Index" in the Cellomics' Neurite Outgrowth BioApplication, which is the percentage of neurons in the well whose neurite lengths are greater than a user-defined threshold.

Because HCS involves the measurements of multiple targets, the simultaneous quantitative monitoring of all these targets gives a more powerful, selective answer whether a cell is a responder. The challenge then is in defining quantitative metrics that take into account the individual response of all the measured targets, to better define the cell as a responder. One method of doing this is to correlate measurements of the different targets. An example is with the HCS analysis of cell cycle. In addition to fluorescent labeling for the cell's DNA content, additional cell-cycle-associated targets can be fluorescently labeled using immunofluorescence, and the correlation of their measurements with the DNA content measurement gives a more informative idea of the cell's cell cycle state. Cellomics' Cell Cycle BioApplication reports the percent of responders for the additional targets with the different ploidy states, as well as the intensity ratio between different cell cycle targets, enabling their states (e.g., phosphorylation state) to be monitored.

Cells within the population might have a range of responses. The heterogeneity of responses provides insight into the nature of the overall response, and rather than being suppressed, should be quantitatively captured. This is possible with HCS, in which individual cells are identified and analyzed, and domain knowledge can guide us in the appropriate way of identifying different subpopulations of cells with their different responses. Some of the ways that we have employed in identifying cellular subpopulations and their responses are:

1. Identifying the percent of cells whose particular measured property is higher or lower than a response threshold (which can either be user defined, or automatically defined from the statistical behavior of a control population) *(1,2)*.
2. Using Boolean logic to combine several raw cellular measurements to define a response event; we have had particular success in identifying different forms and stages of cytotoxicity and cell death with this approach *(2)*.
3. Biology specific responses such as cell cycle phase and cell ploidy *(3–5)*.

4. Categories of HCS Problems

The range of cell biological problems that can be solved using HCS is vast. However, we believe that they fit into a few specific categories based on either the phenotype of the problem or labeling pattern, or on a standard biological response behavior of the cells. In other words, certain types of problems keep reoccurring, even though their biologies might be different. The ability to recognize these HCS problems as belonging to specific phenotypic or biological categories means that for most HCS problems, algorithms directed and optimized toward these categories are all that is needed. The responsibility of an HCS user is that before an analysis strategy is formulated and a specific algorithmic approach can be attempted, recognition and classification of the problem into its biology and phenotypic class has to be done. Only then can the appropriate algorithm with its analysis strategy be used.

Table 1 lists some of the Categories of Problems commonly encountered in HCS, and some of the algorithms designed to solve these problems. These are divided into phenotypic categories in which a certain phenotype of labeling pattern or response is seen, and biological categories in which the cell's biological response is part of a larger general class of cellular responses. The list of categories or the BioApplications in each category is not exhaustive, and we continue to update this list as we encounter further ways to categorize HCS problems, or discover new strategies to solve them; however, this list does represent a large percentage of the HCS problems currently being worked on today. Descriptions of these categories and strategies to solve them are described below.

4.1. Phenotypic Categories of HCS Problems

The phenotypic categories are defined by the appearance of the fluorescently labeled objects and targets in the images. Although their biology might be different, similar phenotypes usually mean similar questions are being asked. Thus, identification and classification of a problem into its phenotypic category in which the analysis has been worked out, facilitates the solving of many HCS assay problems.

4.1.1. Intracellular Intensity Changes

A very common type of problem encountered in HCS is one of measuring a fluorescence intensity change in cells *(2,6)*. In this category of problems the presence (or absence) of a fluorescent dye in a cell reflects a particular cell state. The fluorescent dye can either be an indicator of the cellular environment (e.g., Ca^{++}, pH, membrane potential), or can represent the concentration or amount of a cellular target. A change in the cell's state will cause an increase or decrease in the intensity of the dye in the cell, and in extreme cases might result in a total decrease of fluorescence. An example of an assay, which involves quantitation of intracellular intensity changes, is the monitoring of cell viability. In this particular case, all the cells are identified by their nuclei being labeled with a DNA binding dye (primary object), and the presence of a membrane-impermeant dye colocalized with the nucleus identifies cells that are dead *(2,6,7)*. Quantifying which cells are alive or dead requires measuring the fluorescence intensity of the membrane-impermeant dye in the cells, and if the intensity is above a particular threshold, the cell is identified as being dead. The HCS assay strategy to solve this type of problem is to first identify the primary object, and then quantify the target's intensity in the primary object. Sometimes, this can be run as a one-color assay in which after the primary object has been identified, its intensity is measured; a lack of intensity means that the primary object is not detected.

Table 1
Categories of Problems Encountered for HCS Analysis, and Some of the BioApplications Designed to Solve Them

Phenotypic classifications	Biological classifications
Intracellular intensity changes • All BioApplications	*Intracellular translocation (movement of intracellular objects)* • Target Activation, Compartmental Analysis, Cytoplasm to Nucleus Translocation, Molecular Translocation, GPCR Signaling, Cytoplasm to Membrane Translocation
Counting number of objects • All BioApplications	
Spot analysis • Target Activation, Compartmental Analysis, Spot Detector, Morphology Explorer, Cell Health Profiling, Multiparameter Cytotoxicity, Micronucleus, GPCR Signaling, Multiparameter Apoptosis, Mitotic Index	*Internalization and receptor activation* • Target Activation, Compartmental Analysis, Spot Detector, Morphology Explorer, GPCR Signaling, Receptor Internalization
Colocalization • Target Activation, Compartmental Analysis, Cytoplasm to Nucleus Translocation, Molecular Translocation, Cytoplasm to Membrane Translocation, Cell Viability	*Cell cycle* • Compartmental Analysis, Cell Cycle, Mitotic Index
Cell size and/or shape changes • Target Activation, Compartmental Analysis, Morphology Explorer, Cell Spreading, Cell Motility, Cell Health Profiling, Extended Neurite Outgrowth, Tube Formation	*Neurite outgrowth* • Morphology Explorer, Neurite Outgrowth, Extended Neurite Outgrowth, Tube Formation
Analysis of interconnected tubular objects • Morphology Explorer, Tube Formation	*Monitoring cell health and toxicity* • All BioApplications can be used to monitor cell health, but especially: Target Activation, Compartmental Analysis, Spot Detector, Morphology Explorer, Cell Health Profiling, Multiparameter Cytotoxicity, Micronucleus, Cell Cycle, Cytoplasm to Nucleus Translocation, Molecular Translocation, Cell Spreading, Cell Motility, Cell Viability, Multiparameter Apoptosis, Mitotic Index
Cell movement (fixed end-point assay) • Cell Motility	
and more…	

Measurement of such intracellular intensity changes does not require the spatial information obtained from a microscopy-based approach, and the analysis could be done using non-microscopy-based instruments such as a flow cytometer, in which the fluorescence intensity of each cell is recorded, or even a fluorescence plate reader, in which the intensity of the entire detected field is measured. In HCS assays in which more than one color is used and the target fluorophore has different spectra from the primary object, then the microscope's spatial resolving power is used to first identify and resolve the primary object before detecting the target intensity in it. This approach also enables a subpopulation analysis where one can see the distribution of the target intensity among the primary objects (i.e., cells).

4.1.2. Counting Number of Objects

Another category of problems that arises frequently in HCS involves counting individual objects. The primary objects are typically individual cells or their nuclei, and for this category of problems, the spatial resolving power of microscopy is needed to identify and resolve the individual objects.

The types of biology that fall in this category include cell proliferation assays, migration assays through Boyden chambers, to the simple quantitation of cells containing a particular fluorescent stain *(8–10)*. The key issue here is to identify the primary object, and knowledge of the underlying biology helps in this identification. For example, consider the different types of primary objects involved in the examples shown in **Fig. 1**: cell nucleus, whole cell, cell colony, and angiogenic tube respectively. Biological situations exist in which knowing the number of these primary objects in an image are of interest. However, different strategies (and different algorithms) would be required to identify and count these different types of objects.

4.1.3. Spot Analysis

Another standard type of problem encountered in HCS is one of analyzing spots. In this type of problem, the fluorescent label is sequestered into discreet, punctate objects in the cell (i.e., spots), and the changes in spot properties are what are needed to be quantified. Spot Analysis requires the additional spatial information obtained from a microscopy-based imaging approach. Properties of spots that are of interest include the number of spots, spot intensity, spot size, spot shape, and spot locations inside the cell. One extreme of a spot analysis situation is that a cell only contains one spot defined by the fluorescently labeled target, and the existence and intensity of the spot is the property of interest; this was the assay strategy in early articles in which HCS was used to assay for G protein-coupled receptor (GPCR) activation and internalization *(11–13)*. Another extreme case is that the cell has many spots, and the number of spots and their intensity are of interest; recent articles in which this was the situation being analyzed include biological problems consisting of receptor tyrosine kinase (RTK) activation and internalization, and β-arrestin redistribution on GPCR activation *(1,14)*. Yet another extreme situation is in which the existence of a particular type of spot is a rare occasion, and the property of interest is in identifying and counting the cells which has this spot; this is the basis of micronucleus assays, in which a micronucleus is a special type of spot labeled by the DNA binding dye and adjacent to the nucleus, and its occurrence is a rare event.

The typical strategy in spot analysis is to identify the primary object, and then in an area defined by the primary object (either colocalized with the primary object, or in a region associated or adjacent to it), the spot(s) are detected, and their properties are measured. Spot detection is often enhanced by spatial filters applied during the image processing whose dimensions are tuned to the typical dimensions of the spots in question. For example, in a cell, both the nucleus and its endosomes can be considered as spots; the difference is that the nucleus is a much larger "spot" than an endosome. A spatial filter optimized to enhance identification of either the nucleus or the endosome will enable the algorithm to better analyze the spot under question. The spatial filter size requires domain knowledge, and is usually controlled by a user specified input parameter before applying the algorithm.

4.1.4. Colocalization

Another common type of problem requiring spatial information is one of colocalization. In colocalization problems, a particular region, compartment or organelle of the cell, or a group of intracellular molecules, are identified by a fluorescent label specific for that entity. Then, the target of interest, is identified by a fluorescent probe with a different emission wavelength, and the amount of the target in the cellular region masked by the first probe is measured. A variation on the colocalization problem is that the target is not in the area defined by the primary object mask, but in an area associated with or adjacent to it.

A common colocalization type of problem encountered in HCS is the translocation of an activated transcription factor from the cytoplasm to the nucleus. This type of problem typically involves a fluorescent-labeled nucleus as the primary object, which identifies individual cells as well as the cytoplasmic and nuclear areas between which the transcription factor's translocation occurs. Various labeling strategies can identify the transcription factor, and immunofluorescence

is a common labeling option. Unstimulated cells have fluorescent cytoplasm, and nuclei relatively devoid of signal. The translocation event results in the nuclear signal increasing at the expense of the cytoplasmic signal. The analysis strategy for this problem involves measuring the transcription factor's signal colocalized with the nucleus, which has been identified by a nuclear specific fluorescent dye, and also simultaneously monitoring it in the cell's cytoplasm (**Figs. 1–6**) ***(1,15–17)***. The cytoplasm is usually defined as an annular region outside the nuclear mask; even though the nuclear label does not cover the cytoplasmic region, its existence allows definition of the cytoplasmic region adjacent to it. Understanding that the target has moved from the cytoplasm to the nucleus, thus causing the opposing intensity changes in these two regions of the cell, enables a directed analysis strategy in which the two distinct cellular regions (cytoplasm and nucleus) are first accurately and optimally identified, and then the translocation event is most sensitively and robustly measured by either the difference or the ratio in intensities between these two distinct regions (**Figs. 1–6**). Not understanding the cell biological process being measured might result in the targeted identification of the cells' distinct cytoplasmic and nuclear regions not being made. Thus, although the transcription factor's intensity would be measured, the actual translocation event would not be as sensitively captured because the intensity difference or ratio between the two cellular regions would not be done. Thus, domain knowledge of this biology is needed to devise the appropriate measurements for the translocation process.

This is an example of a nuclear colocalization problem, but colocalization problems are not restricted only to nuclei; other compartments, including the whole cell or even a multicellular assemblage, can also be the primary object. Cellomics' Compartmental Analysis BioApplication was designed to identify multiple different regions of a cell based on the area covered by the primary object, but some of these regions do not necessarily overlap with the primary object, and might often be adjacent to it ***(1)***.

4.1.5. Quantifying Cell Size and Shape Changes

A standard cellular response to stimuli or changing conditions is that either the whole cell, or cellular components (such as organelles or cytoskeleton) might change in size or shape. Size and shape changes are subsets of a larger problem of quantifying the spatial rearrangement of the detected entities. As HCS is based on microscopy, it is well suited to quantifying these sorts of morphological and size changes, and related changes in spatial arrangement ***(2,6,7,18)***. The entity whose morphology changes are being reported is either the primary object (e.g., the whole cell) or components of the primary object. The key in this sort of analysis is to again first identify the primary object, and then to identify the targets and cellular entities associated with the primary object whose analysis is of interest. Once these have been identified, it is relatively straightforward to measure their different morphological and size features, and other spatial information.

In a cell biological context, spatial changes resulting from stimuli can really occur over three different spatial dimensional scales: subcellular morphology, whole cell morphology, and multicellular morphology. Often, responses to stimuli can occur over all three scales and are correlated. We, thus, designed our algorithms in this category to quantitatively report phenomena that change over the multiple spatial scales. Cellomics' Morphology Explorer BioApplication reports spatial rearrangements over all three spatial scales. Features reported for whole cell morphology include cell shape, dimensions, orientation, and extent, as well as quantitation of cellular outgrowths. Features reported at the subcellular level include the intracellular location and amounts of macromolecules or discrete objects, their intracellular arrangement including a range of different texture measurements, properties of intracellular spots or fibers, and the shape and dimensions of major intracellular compartments such as the nucleus or Golgi. Multicellular features reported include the shape and dimensions of multicellular assemblages such as colonies or multinucleated cells, the proximity and spacing of similar and dissimilar cells or colonies, and the number of cells in such assemblages.

An example of a problem in this category is to measure the effects of a drug that affects the cytoskeleton. Such compounds cause changes both in the whole cell morphology as well as in the structure and arrangement of the cytoskeleton. Thus, the strategy employed is to use a whole cell stain to identify the cell as the primary object, measure its morphology, and then to specifically identify and measure the cytoskeletal fibers amidst any background fluorescence (**Fig. 1**). In **Fig. 1**, several of the cells are touching; so advanced segmentation techniques were employed to identify the distinct areas covered by the individual cells, even if they were touching. Understanding the biological situation would allow one to recognize that segmentation to separate and resolve touching cells was needed in this particular biological situation, but would not be needed in other situations in which the object being analyzed was a multicellular assemblage. Identification of the cytoskeletal fibers first required recognition that the fibers, in an associated fluorescent channel, were contained within the area covered by the individual cell, and then to recognize the fibers among the fluorescent background. Knowledge that cytoskeletal fibers were the targets that had to be identified and analyzed directed the algorithm to selectively detect such structures; an undirected approach might not have detected the fibers as optimally as the directed approach.

Another example in this category is the analysis of cell colonies. **Figure 1** shows a colony of embryonic stem cells growing on top of a mouse fibroblast feeder layer; all cells' nuclei were labeled with Hoechst 33342, and the stem cell colonies are the primary objects. Directed analysis using Cellomics' Compartmental Analysis BioApplication was done in which the nuclear label was used to identify the colony. A size exclusion principle was applied to ignore small objects (i.e., mouse fibroblast nuclei), and to only analyze large objects, which were the stem cell colonies. Lack of domain knowledge might cause all cells to be analyzed, thus obscuring the actual object of interest, the stem cell colonies. Once the colonies were identified, the number of Hoechst 33342 labeled nuclei, identified as spots within the colony, were counted as a measure of colony size, and in additional channels, October 4 labeling allowed identification of the number of pluripotent cells in the colony.

4.1.6. Analysis of Interconnected Tubular Objects

For many of the problems encountered in HCS, the primary objects are self-contained entities such as cells, nuclei, and colonies. However, the object of interest could be an interconnected structure with dark areas (i.e., holes) within the fluorescent label. An extreme case of this is a tubular network, which is made of many cells, and the primary object is the entire multicellular tube. Examples in this category include angiogenic tube formation (**Fig. 1**), differentiated myoblasts forming myotubes, and tangled interconnected webs of neurites ***(19,20)***.

In angiogenic tube formation, individual endothelial cells associate with each other to form a tubular, branched capillary network. These connected tubes are the primary objects. Analysis of tubes is a specialized case of morphological analysis in which once the primary object (i.e., the tube) has been identified, questions asked of it include its length, width, area, number of branch nodes, and spacing between branch nodes. Domain knowledge leads one to recognize that the individual cells are associated with each other in a linear, interconnected tubular network, so the directed algorithm applied has to trace and connect the cells making up this network in order to identify the entire primary object. If the domain knowledge of tubulogenesis was not there, the analysis strategy might miss the association of the cells into tubes, and thus might not report the tube properties of interest.

4.1.7. Cell Movement

Another cellular response that can be quantitatively analyzed by HCS assays is cell motility, and there are several ways that have been designed to assay this behavior by HCS. Because motility is a dynamic process, one way of analyzing it is to take a sequence of images over time of the motile cells, and then using the spatial coordinates over time, compute motility parameters ***(6)***. However, HCS assay methods of quantifying cell motility have been developed in which the cells on the sample plates being imaged are fixed. One assay technique is to use modified

Boyden chamber inserts for microtiter plates, and to count the number of cells that migrated through the Boyden chamber membrane; this reduces the analysis problem to one of just counting the number of cells *(8,21)*.

An alternative fixed end-point assay technique for cell motility is based on the method of Albrecht-Buehler et al. *(22)*. In an adaptation of this method, cells move on a lawn of fluorescent beads clearing the beads as they move and leaving behind dark tracks; the track areas are proportional to the cells' mean-squared displacement movement over time, and is the primary object that is quantified *(21)*. In this case, the primary object is neither the fluorescently labeled entities (the lawn of fluorescent beads), nor the cells, but the dark tracks that mark the area of the cell's movement. This is akin to analyzing a structure in a negative image. Once the dark tracks or regions have been identified, their properties (such as their length and area) can be measured. The cells in the tracks can be detected by other dyes, and the number of cells per track can also be reported.

4.2. Biological Categories of HCS Problems

HCS problems can also be classified by the broad categories of their biological response. This classification facilitates identification of particular solution strategies. Many of these solutions involve recognition of a certain phenotypic response, which fits into one of the categories described in the previous section, thus, further facilitating the solution of the problem.

4.2.1. Intracellular Translocation

A common response of cells to stimuli in HCS assays is that the fluorescently labeled cellular target moves from one region of the cell to another. In this category of problem, understanding of the source and destination of the target's movement is necessary. Then the assay strategy involves identifying the source and destination regions, and quantifying the target's presence in them (e.g., intensity). Arithmetic operations (e.g., differences, ratios) between the amount of target in the two regions further facilitates quantitative assaying of the translocation event. One of the earliest, and most common examples of this cellular response subjected to HCS analysis was the translocation of a protein from the cytoplasm to the nucleus of the cell *(1,15–17)*.

For translocation events between distinct compartments or regions of the cell, algorithms such as Cellomics' Compartmental Analysis or Molecular Translocation BioApplications can be used to identify the regions, and then measure the translocation (as discussed in **Subheading 4.1.4.**). If one of the regions where the target needs to be detected is made up of a punctate pattern (i.e., spots), then a spot analysis approach, as previously discussed, could be used. Sometimes, the region might involve translocation to or from the plasma membrane, and this requires identification of the cell surface. One way to do this is to directly label the cell surface in a colocalization type of approach. An alternative is to recognize that the cellular image is a two-dimensional projection of the entire cell, and that if the label is not present on the cell's surface, then the area of plasma membrane over the nucleus might not have any label, giving the cells an area devoid of labeling where the nucleus. When the target translocates to the plasma membrane, the patch above the nucleus gets filled in and the dark region above the nucleus disappears. Thus, this allows an analysis strategy similar to the cytoplasm to nucleus translocation approach, because even though the translocation is between the cytoplasm and the cell membrane, the labeling pattern is the same as a cytoplasm to nucleus translocation situation, allowing the same quantitation technique to be used.

4.2.2. Internalization and Receptor Activation

One form of translocation is the internalization of a receptor from the cell surface. Internalization of signaling receptors, such as GPCRs and RTKs, occurs after the receptor's activation by an agonist. GPCRs internalize and then get recycled back to the plasma membrane as part of their desensitization and resensitization steps, whereas RTKs often get internalized as a part of their down-regulation. Nevertheless, the internalization of these signaling receptors indicates

that their activation by an agonist has occurred, and, thus, can be used as a surrogate assay for receptor activation *(1,11–14)*. The internalized receptors in endosomes typically have a punctate appearance, and thus the problem becomes one of spot analysis.

4.2.3. Cell Cycle

As described earlier, HCS can be used for cell cycle analysis. Cell cycle analysis has been done in several different ways including: (1) analyzing the presence of a specific cell cycle indicator to determine whether the cell was in a particular cell cycle state (e.g., the presence of phosphorylated histone H3, detected by immunofluorescence, indicates that the cell is mitotic); (2) measuring the DNA content of the cells from a DNA binding dye to determine the cell's ploidy; and (3) to combine and correlate the cell ploidy measurements with other cell cycle associated proteins for a better indication of the cell's particular state *(3–5,9,23)*.

As discussed earlier, identification of nuclei is enabled by labeling with a DNA binding dye, and the DNA content is obtained from the total intensity of the dye in the nucleus. Population analysis of these intensities over all the nuclei defines the categories of different ploidy of cell cycle states (i.e., cells in G1, S, or G2), and then each nucleus can be assigned a state. Identifying and measuring nuclear intensity is straightforward, but additional analysis in identifying and assigning cell cycle states is needed. Several directed algorithms, including Cellomics' Cell Cycle BioApplication, have been introduced to provide this enhanced analysis. Measurement of other cell cycle targets usually involves measuring the intensity or spot analysis of the targets, and their correlations can either be built in to the analysis packages, or can be done offline with third party software.

4.2.4. Neurite Outgrowth

Often, particular cells such as dendritic cells and neurons have processes. The item of interest are these processes, and the questions asked of them include the number of them per cell, their length, and their degree of branchedness. We developed several different directed algorithms to quantify neurite outgrowth, and which can be applied to this specific type of morphological problem *(24–28)*.

We have implemented three approaches for quantifying neurite outgrowth types of problems. In the approach offered by Cellomics' Morphology Explorer BioApplication, the neuron is the primary object. Once the neuron has been identified, the algorithm identifies and traces any processes emanating from the cell body. It then reports size and shape properties of the cell body, as well as the number of processes emanating from it, and their lengths.

An alternative cell-based approach is employed by Cellomics' Extended Neurite Outgrowth BioApplication. This was designed to be used in mixed cell cultures in which not all of the cells might be neurons with neurites, or a neuron of the correct type. In this situation, the primary object label is still a neuronal stain, which also contains a valid nucleus. The presence of both the neuronal specific stain and a labeled valid nucleus identifies the primary objects as being valid neurons, and it is only on those that the algorithm specifically identifies and traces neurites.

A third approach is where neurite outgrowth is reported at the field level. This approach is often used when the situation involves an interconnected mat of overgrown neurites. In this case, because of the overgrown neurites, it is often difficult to match neurites to their cell bodies. One way to solve this is by using Cellomics' Neurite Outgrowth BioApplication, which is a fast, field-based approach in which accurate identification of the cell body for each neurite is not needed, and an overall measure of neurite outgrowth for the field suffices. The situation is also analogous to the phenotypes seen in a tube formation situation, and thus a similar analysis strategy can be applied. The analysis strategy is to treat the entire mat of tangled neurites and cell bodies as the primary object, and then report the length of the web of neurites, the number of branch or cross points, and the length of neurite segments between branch and cross points.

4.2.5. Monitoring Cell Health and Toxicity

An area of growing popularity for HCS is in the monitoring of cell health and cytotoxicity. Although any HCS assay that reports on the physiological state of cells could be used as a cell health or cytotoxicity assay, there have been some specific applications of HCS toward monitoring cytotoxicity and apoptosis *(2,6,7,18,29)*. These applications have combined and correlated measurements several of the phenotypic classes discussed such as nuclear size changes, with cell intensity changes from a membrane-impermeant dye, to spot analysis monitoring the changes in mitochondrial transmembrane potential *(2,6,7,18,29)*. Measurement and correlation of multiple different properties related to cell health measured simultaneously in the cell gives a better understanding of the cell's health, and also enables uncovering the sequence of events leading to a cell's death in response to a toxic insult. This class of assays is discussed in other chapters in this book.

5. Practical Utilization of Directed Algorithms to HCS Problems

Cytoplasm to nucleus translocation is one of the earliest HCS problems worked on, and one of the first directed algorithms released by Cellomics *(15)*. In this section, we describe it in detail, as a practical example of a directed algorithm. The cytoplasm to nucleus translocation algorithm measures translocation events by quantifying the relative distribution of target fluorescence intensities between the cytoplasm region and the nuclear region of a cell (**Fig. 2**). Measuring the intensity within nucleus and then comparing it with the intensity of cytoplasmic region provides a simple and very robust way to assess quantitatively the degree of cytoplasm to nucleus translocation. The issue remaining is how to specifically measure the intensities in these two regions.

Let's consider a real life example with the goal to characterize the activation and nuclear translocation of the transcription factor nuclear factor (NF)-κB in HeLa cells. HeLa cells plated in 96-well microplates were treated with two concentrations (Min/Max) of IL-1α, fixed and stained using the reagents and protocol in Cellomics' NFκB Activation HitKit® HCS Reagent Kit (**Fig. 3**). The algorithm takes advantage of two-color fluorescence labeling of the cells (**Fig. 4**). The image of the same field is acquired twice using different excitation-registration channels: one-nucleus channel (blue fluorescence), the other-target channel (green fluorescence). First the primary objects (nuclei) need to be identified. In a typical case a limited number of image processing procedures is needed. Those include background correction, smoothing and intensity thresholding. **Figure 5** shows an overlay of the object mask created as a result of applying of these procedures. In some cases additional steps like object segmentation, border touching object removal, and so on might be required.

After identification of the primary object, target identification is performed. It might consists of similar processing steps: background correction, smoothing and intensity thresholding and, if necessary, segmentation (**Fig. 6B**). The next step is to create measurement masks corresponding to the already defined regions of interest: nucleus and cytoplasm. We have found that the best way to accomplish this is to use the nucleus mask and modify it to derive masks that correspond to the areas (regions) of interest (**Fig. 6**). The mask that covers the nuclear region of interest (called CIRC), can be created by simple erosion of the nucleus mask (**Fig. 6A**). At the same time the mask that covers the cytoplasmic region of interest (called RING), can be created by dilation of the nucleus mask and applying logical XOR operation to the dilated and the original nucleus mask (**Fig. 6B**). To move the RING mask further away from the nuclear region, the original nucleus mask must be also dilated. The size of dilation must correspond to the distance in pixels the inner edge of RING to the nuclear region. Thus, the CIRC and RING masks, their size (width) and location relative to the nucleus edge, can be fully controlled through the combination of erosion/dilation applied to the nucleus mask. In some cases, when cells are elongated, the cytoplasmic region might become very narrow and as a result the RING mask can fall (extend) beyond the target and has to be confined to the target mask (**Fig. 6F**).

Table 2
Intensity Measurements (Arbitrary Units) Made From the Target Channel: Before and After Translocation

	Avg. inten CIRC	Avg. inten RING	Avg. inten diff CIRC–RING	Avg. inten ratio CIRC/RING
Before	760	1200	–440	0.63
After	1840	640	1200	2.85

After CIRC and RING masks are created measurements of intensity can be made from a target by simply calculating the aggregate intensities of the target image under the CIRC and confined RING masks (**Fig. 6C,F**) and then computing the average pixel intensity within the CIRC and confined RING masks. Typically the average pixel intensities under the CIRC and RING masks, but not the aggregate intensities under the original nucleus mask and target mask is used to measure the degree of the translocation response.

Visually the process of cytoplasm to nucleus translocation can be described as an increase of transcription factor concentration within the nuclear region and its decrease with the cytoplasmic area around the nucleus. In image analysis terms the concentration of the labeled material (transcription factors) within a particular region is nothing but the average pixel intensity within that region. Thus, comparing the average pixel intensity of the cytoplasmic area around the nucleus (RING) with its average pixel intensity inside the nuclear region (CIRC) provides a simple way for quantitative description of cytoplasm to nucleus translocation process. As a metric for the translocation process, typically two measures are used: the difference or the ratio between average pixel intensity under CIRC and RING masks (**Table 2**). The use of each has its pros and cons. The difference is insensitive to the nonspecific background intensity, but produces different (inconsistent) results if the intensity of illumination source changes or the excitation illumination is spatially nonuniform. The ratio, vise versa, is insensitive to the intensity of illumination source change or to spatial nonuniformity in the excitation illumination but produces poor (inconsistent) results if nonspecific background intensity is not removed.

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