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PERSPECTIVE

Image-based chemical screening

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Technological advances have made it feasible to conduct high-throughput small-molecule screens based on visual phenotypes of individual cells, using automated imaging and analysis. These screens are rapidly moving from being small, proof-of-principle tests to robust and widespread screens of hundreds of thousands of compounds. Automated imaging screens maximize the information obtained in an initial screen and improve the ability to select high-quality leads. In this Perspective, I highlight the key steps necessary for conducting a high-throughput image-based chemical compound screen.

Visual assays, in which cells are fluorescently labeled and observed using a microscope, are distinguished among other types of assays in the amount and quality of information acquired in a single assay sample. Compared with biochemical assays, they probe a chemical's activity on a cellular phenotype rather than simply testing for binding to a particular isolated protein that may be, in the end, less physiologically relevant. Image-based assays also compare favorably to other types of cell-based assays (for example, reporter assays using plate readers) because multiple features of the cell's status and health can be observed, including very general phenotypes, such as overall cell health, and very specific phenotypes, such as localization of a particular phosphorylated form of a signaling protein. Even a single label allows multiple features of the cell's state to be measured. For example, a simple DNA stain allows counting the cells, plus measurement of each cell's DNA content and morphology, which together indicate the cell's phase in the cell cycle and its apoptotic state. Image-based assays are almost always further "multiplexed" by using multiple fluorescent labels to report on several components of the cell simultaneously[1]. Further, most image-based assays collect information from individual cells, which is often necessary to accurately reflect the underlying biological process—behavior that may be hidden in whole-population measurements[2,3]. This is particularly the case when only a subset of cells is expected to show the phenotype, for example when a cell mixture has been used for the screen. The rich information that can be collected from individual cells has led image-based assays to be dubbed "high in information content" or "high-content."

However, until roughly ten years ago, visual assays were nearly always very low-throughput, requiring tedious manual collection of images followed by visual inspection of tens of thousands of images. Since then two major technologies, automated microscopes and cell image analysis software, have gained traction and rapidly made image-based assays compatible with high-throughput screening (HTS) of small-molecule libraries; the resulting process has earned the name "high-content screening" (HCS)[4]. The pharmaceutical industry has provided a user base large enough to push the development of the systems needed for image-based screening; indeed, though companies often do not publish the results of their large screens, there are notable exceptions[5–9], and they remain among the largest users of these technologies[10]. The explosion of high-throughput RNA interference experiments to determine gene function has also driven the maturation and spread of these technologies[11–13]. The result of these developments is that we can now automatically acquire and score images in large-scale screens. However, most published image-based chemical screens have been at the assay development stage, and the major use of the technology, at least in the pharmaceutical industry, is at the secondary-screen stage. Indeed, image-based assays are still time consuming to conduct and interpret, so instead a plate reader–based assay ought to be pursued if it would sufficiently reveal the primary readout of interest and if additional phenotypes from the primary sample are of little interest. Still, image-based primary screens of 10,000–100,000 compounds are rapidly becoming more common, and their power to maximize the information obtained in an initial screen and improve the ability to select high-quality leads indicates that they will soon be a standard primary screening modality.

Here, I overview the current state of each key component necessary for implementing a successful image-based chemical screen (**Fig. 1**), and point to available resources and further reading. Although I have focused here on cell-based screens because they are by far the most automated, it should be noted that several efforts are under way to develop methods for the automated sample preparation, imaging, and (most challenging of all) image analysis of whole organisms, such as zebrafish[14], *Caenorhabditis elegans*[15] and *Arabidopsis thaliana*[16].

Biological assay development

Image-based chemical assays begin as standard microscopy assays, in which, typically, the localization or amount of a fluorescently labeled protein or the morphology of the cell responds to a biological change of interest. Establishing such an assay might involve months of perfecting immunostaining protocols, tagging a protein of interest with green fluorescent protein or its various-colored derivatives, engineering a cell line to respond to stimuli appropriately, or developing methods to extract primary cells. Developing a suitable assay to probe the phenotype of interest is critical to the quality of the screen's data. 'You get what you screen for', so ideally the assay system should probe the desired biological outcome as closely as possible rather than pursuing artificial proxies, to the extent that it is practical. For example, creating realistic cell-based models for disease and assaying for reversal of the phenotype is perhaps the best type of assay, but this is much less common than measuring the level or localization of a signaling molecule thought to be in the disease's pathway.

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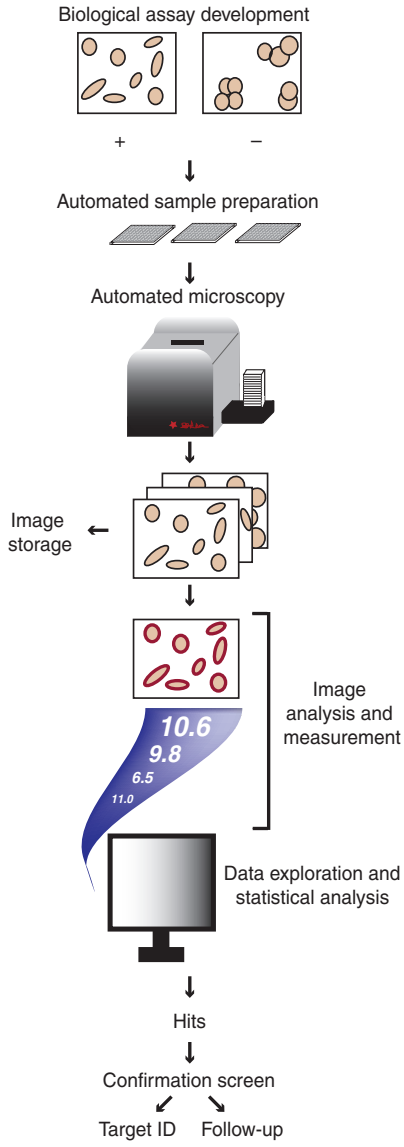

Figure 1 The key steps necessary for conducting a high-throughput image-based chemical compound screen.

Once the assay is robust, the scale-up process begins to automate and miniaturize the assay to a 96-, 384- or 1,536-well plate format to conserve labor and reagents. Higher density formats have been developed (for example, a cell microarray format[17]), but have yet to become routine. A helpful general guide to assay development (though it does not yet cover image-based assays) has been developed by Eli Lilly and Company and the US National Institutes of Health (NIH) Chemical Genomics Center (http://www.ncgc.nih.gov/guidance/index.html). Also, this issue includes a Review from Inglese *et al.* that provides a guide to developing assays for high-throughput screening and is particularly aimed at scientists that are new to the field[18].

Image acquisition and storage

After an assay has been optimized and sample preparation has been automated, the samples are imaged on automated microscopes. Initial microscopes for high-throughput image-based screening were simply standard fluorescence microscopes with automation added to handle multiwell plate formats, including automatic stage movement and focusing. First commercialized in 1999 (the ArrayScan from Cellomics), the instruments have become increasingly automated. Though options have thinned after the acquisition of many smaller companies, a wide variety of instruments are still available that differ in speed, image quality/resolution and cost. Other commercial instrument options include integration with automation (for example, automatic plate handlers so that stacks of plates are automatically loaded, and pipettors for adding reagents during the screen), environmental control of heat and humidity for live experiments, and ability to acquire confocal images. The ability to export images in a standard image file format (for example, the OME-TIFF format, http://www.loci.wisc.edu/ome/ome-tiff.html) is another critical feature that is necessary for using third-party image analysis and data exploration tools, and even for convenient viewing of images from a screen. Commercial options have recently been reviewed, including listings of the available instruments and their features[19–22]. Today, most active screening groups use instruments that are sufficiently automated to handle dozens of plates without user intervention. Most of these instruments are reliable and mature technologies. The only common headache, aside from software/network/database integration, is unreliable automatic focusing for certain sample types and plate types, and with certain instruments.

Avoiding prepackaged commercial instruments, several academic groups have made the effort to construct complete image acquisition systems from component parts to meet cost and/or flexibility demands[23–25]. These instruments typically produce images as high in quality as those produced by commercial instruments, depending on the quality of the component parts that are used. The trade-off is rather the labor and expertise required for assembly. Beyond these, an open-source software package in development may change the landscape of this field in the future: MicroManager software controls microscope hardware, theoretically enabling convenient construction of a build-it-yourself high-throughput microscope (μManager, http://www.micro-manager.org/). This open-source package may allow a lower cost of entry such that automated imaging becomes more widely available. It should also enable analysis of unusual sample formats (for example, cell microarrays[17]) or unusual experimental protocols (for example, three-dimensional or time-lapse imaging).

Along with image acquisition, a solution for image storage must be developed. Typical screens collect several images per sample; with a typical image requiring 1 MB of storage space, a 100,000-compound screen requires ~500 GB (0.5 TB) of disk space. Storing the images within a database purchased along with a commercial system typically works well, but it can be problematic to integrate several systems, even from the same vendor, or to expand the database itself. In some cases, organizing images in a simple, file-system-based hierarchy may be preferred. Although the open-source software project Open Microscopy Environment (http://openmicroscopy.org/) is not widely used for large numbers of large screens, it has proven useful for the storage and organization of images in smaller-scale laboratories.

For many scientists, the simplest solution for both sample preparation and image acquisition may be to make use of one of the many existing screening centers with this technology. In the United States, image-based screening capabilities are available via some of the NIH-sponsored Molecular Library Screening Center Network sites (http://mli.nih.gov/mlscn/descriptions.php), the National Cancer Institute (NCI)-sponsored Initiative for Chemical Genetics (http://www.broad.harvard.edu/chembio/icg/) and the Genomics Institute of the Novartis Foundation (http://www.gnf.org/collaborations/academic-screening-program). Furthermore, funding specifically for chemical screens is available from the NIH (http://mli.nih.gov/mlscn/resources.php). In addition, the Society for Biomolecular Sciences maintains a partial list of academic

screening centers throughout the world (http://sbsonline.org/ascd/index.php).

Image analysis

After acquisition, image analysis is required to extract quantitative measurements from the images for each sample. Image analysis for common phenotypes in well-behaved (non-clumped) cell types has been well solved by commercial software[5–7,9,26,27]. This software is usually sold in a bundle with the image acquisition hardware, but more recently it has become available from third-party software vendors[28,29]. Cell counting, protein expression, translocation (including translocation from the cytoplasm to the nucleus and translocation of proteins from a smooth distribution to a speckled distribution), and neurite outgrowth, which are assays frequently used in the drug-development industry that has funded much of this technology development, are readily measured by commercially available software (**Fig. 2a**). Furthermore, commercial software can sometimes be 'tricked' into measuring the phenotype of interest by adapting the standard applications to a different purpose. For example, to measure cholesterol accumulation in images of cells stained with filipin A, a high threshold identified the lysosome-like storage organelles (LSOs) while a low threshold identified the area occupied by cells, thereby enabling intensities to be appropriately normalized for cell size[30] (**Fig. 2b**).

However, analyses that cannot be achieved with the existing applications in commercial software remains challenging[31]. Some investigators have turned to tedious manual inspection of images for scoring; example phenotypes include cytokinesis, wound healing, nuclear localization, Golgi-to-plasma membrane transport and aggresome formation[32–36] (**Fig. 2c**). Other researchers have developed custom scoring software, programmed from scratch or by developing macros in an existing open-source tool such as ImageJ (http://rsb.info.nih.gov/ij). This usually requires months of development and validation; example phenotypes include focal adhesions, centrosome duplication, cell cycle progression, apoptosis and a wide variety of morphological features of cells, nuclei and organelles in combination with various fluorescent labels[8,23,37–41] (**Fig. 2d**). Unfortunately, these custom solutions are rarely reused for other screens because they are so specifically tailored to particular experimental conditions.

Clearly a more flexible solution to image analysis would increase the speed of assay development for high-throughput image-based screens. Academic groups, including mine, have been collaborating on a pair of more general, open-source software tools that aims to simplify the process. Rather than designing a custom

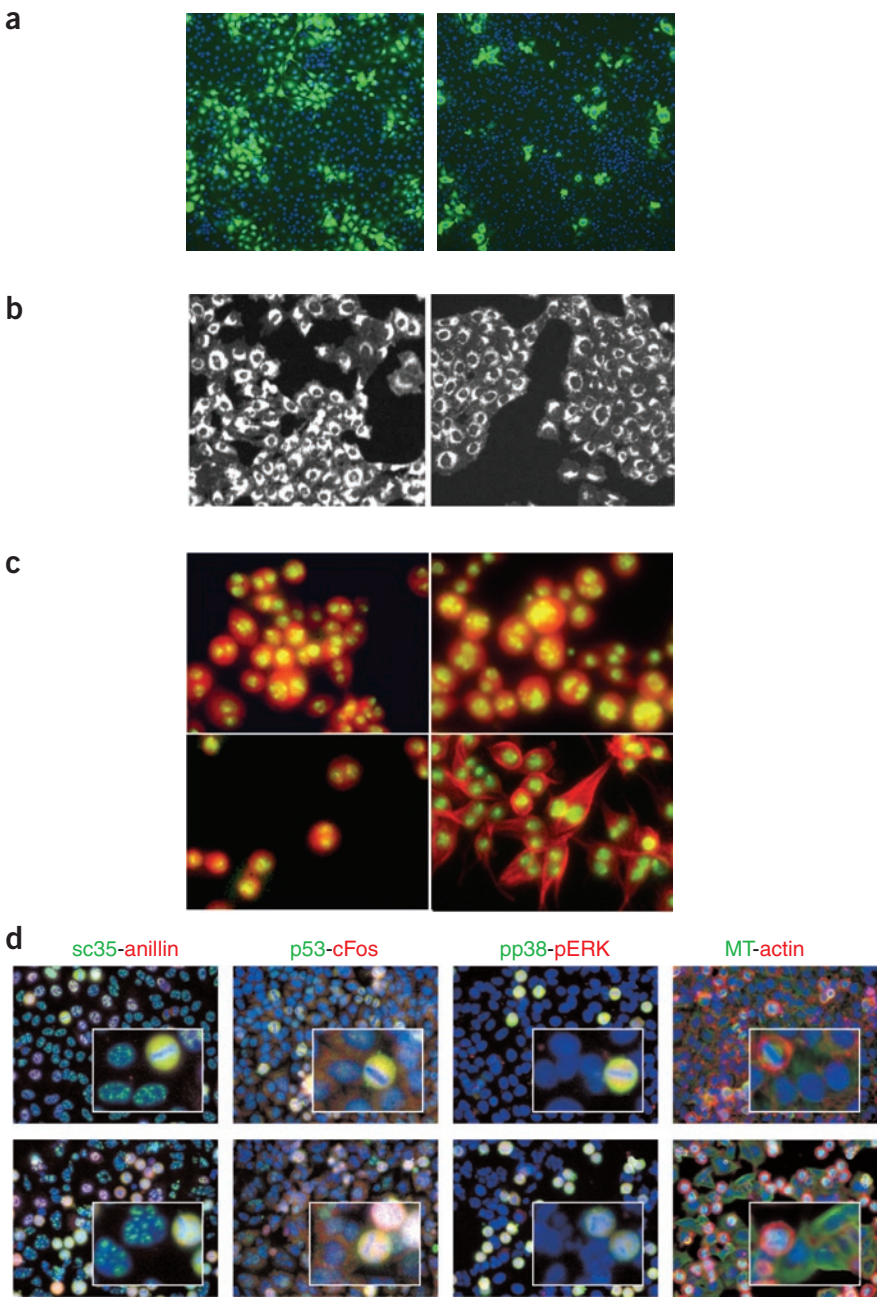

Figure 2 A sampling of image-based phenotypes scored in recent screens. (**a**) Negative (left) and positive (right) controls in an assay for gap junction blockers, scored using a commercial software algorithm to identify dye-positive cells that have received the dye, through gap junctions, from donor cells that were preloaded with dye. 486,000 compounds were screened[6]. Images are reproduced with permission from ref. 6. Images were provided courtesy of J. Li. (**b**) Negative control (left) and positive sample (right) in an assay for inhibitors of cholesterol accumulation in LSOs. In these images stained with filipin, bright regions in each image are the LSOs, whereas dimmer regions show the whole cell area. Commercial software was used to score images. 14,956 compounds were screened[30]. Images are reproduced with permission from ref. 30. Images were provided courtesy of F. Maxfield. (**c**) Four example phenotypes in an assay for cytokinesis defects (particularly binucleation), scored by visual inspection and automated analysis. 51,000 compounds plus 20,000 double-stranded RNAs (RNA interference reagents to knock down each gene's expression) were screened in *Drosophila melanogaster*[32]. Images are reproduced with permission from ref. 32. Images were provided courtesy of U. Eggert. (**d**) Negative (top) and positive (bottom) controls in assays for various readouts of cellular states, as indicated by the stain labels across the tops of the images. 100 compounds were tested across dose-response curves (13 doses) in these four separate assays. A magnified view of selected cells in mitosis and interphase is shown in the inset. Images were scored using custom-developed software[40]. Images are reproduced with permission from ref. 40. Images were provided courtesy of S. Altschuler.

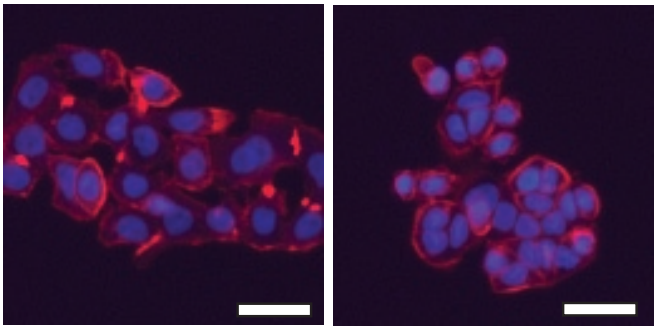

Figure 3 An example of an unusual, subtle phenotype recently screened in our group and scored automatically using CellProfiler for image analysis and CellVisualizer for machine learning–based automated scoring (raw screening images are shown). The cells in the sample on the left have a higher percentage of cells with a bright dot of actin staining (red, phalloidin) as compared with the control cells on the right. Scale bar, 20 μm.

image analysis routine for each new phenotype of interest, we have used CellProfiler cell image analysis software as a general tool to identify cells and their subcellular compartments, and to measure hundreds of features for each cell[42] (http://www.cellprofiler.org). Biologists then spend a few hours using CellVisualizer data exploration software to select a few hundred example cells having the phenotype of interest (http://www.cellvisualizer.org, in development and soon to be released open-source). Behind the scenes, machine learning algorithms determine rules based on the CellProfiler-measured features, thus enabling automatic, accurate scoring of complex phenotypes within a single day. A variety of unusual, subtle or rare (low-penetrance) phenotype screens have been successful in my group and with collaborators (**Fig. 3**). Although setting up and learning these software packages requires a few weeks of initial effort, and some knowledge of image analysis is very helpful for optimal results, the release of these experimental methods in the form of user-friendly software tools will enable screens for previously 'impossible' complex phenotypes and shorter assay development time for each new assay.

The main parameters in choosing image analysis software are data quality, speed (often inversely related to data quality), flexibility to assays of interest, cost and ease of use—particularly ease of integration of the software with the rest of the screening pipeline. I will address the first two here, having discussed flexibility above. Despite the public availability of standard image sets (http://ravkin.net/SBS/Invitation-Algorithm-Comparison.htm) on which to test software for reporting the resulting Z' factor (a measure of assay quality), very few vendors have done so. Currently, CellProfiler holds the record for the highest published score on one of these image sets[42]. Consumer demand for objective evidence of the quality of the software has not yet driven vendors to conduct these tests and make the results publicly available. Regarding speed, commercial software that is bundled with the image acquisition instrument tends to process images quickly enough to keep up with image acquisition; thus, analysis is performed 'on the fly'. Software such as CellProfiler and some commercial third-party software packages instead perform image analysis at a separate stage, after image acquisition. This requires effort to integrate the screening workflow, and, in the case of CellProfiler, a computing cluster is required to complete a full screen's image analysis (~1–5 min per image) in a matter of hours and thus avoid a bottleneck.

Data analysis and exploration

Once the phenotypes have been scored quantitatively, statistical analysis is the next step. Though tools for this purpose are beginning to appear[43,44], proper statistical techniques for scoring screens of any type are still widely debated. As has been the case in microarray data analysis, this should settle into user-friendly software and generally accepted methods over the next few years. Two major areas in which the field has not yet reached consensus are (i) how to correct biases in the data, which are generally due to plate-to-plate variation or position-within-the-plate anomalies such as edge effects, and (ii) how to select hits from the screen, which is often decided based on the number of compounds the researcher is willing to pursue in follow-up work, rather than on a rigorous statistical basis. Most commonly, a bioinformaticist or statistician is consulted to assist in the analysis of data using statistical software, such as the free software package R (http://www.r-project.org). At the least, this analysis should examine the assay readouts for reproducibility between replicates, spatial and temporal biases in the data (both across and within plates), and assay quality (usually a Z' factor of at least 0.2, and preferably greater than 0.5). Assays not meeting basic criteria should either be reoptimized and repeated, or, if borderline, normalized and corrected statistically where possible.

Beyond statistical analysis, data exploration is another underdeveloped area. Image-based assays inherently contain far more quantitative information about each cell than could possibly be visualized and explored by a researcher. Most of this information is ignored and instead the phenotype of interest is measured from one or a few numerical outputs from the screen. Furthermore, individual cell data within each well are usually merged by taking a mean or median of the cells' values from the well, or by choosing the percentage of cells above a particular threshold. In most cases, commercial image analysis software does not produce the full spectrum of information available in the screen—which is understandable, given the goals of speed and simplicity.

Still, methods for storing and harvesting some of this normally discarded useful information are rapidly developing, to the great advantage of screeners. For example, the fact that many phenotypes are measured for each cell in each image means that virtual secondary screens are possible for readouts other than the primary assay readout. This allows hits as determined by the primary readout of the screen to be further characterized for secondary readouts, using the original screening data. For example, if a DNA stain has been included in the screen, hits in the assay can quickly be categorized as to whether they affect cell proliferation (by looking at cell counts) and cell cycle progression (by looking for abnormal DNA content via cell cycle histograms)[45]. Several studies have done beautifully in-depth analyses of a small number of compounds, where rather than arbitrarily defining the 'primary' and 'secondary' phenotypes, they have instead explored multiple phenotypes up front[37,38,40]. Less scientifically important but practically quite useful is the ability to use data exploration tools to automatically remove false positives using measured features of the cells and/or images. This has traditionally been done by visual inspection[27], but it is possible in our experience to distinguish and eliminate cytotoxic compounds, fluorescent compounds, and out-of-focus images based on measured features (unpublished data).

As mentioned above, although methods are gradually becoming available to properly make the most of this useful information[46], no current tools meet all these needs for data exploration and analysis. As a result, most analyses have used custom tools[8,37,38,40]. Still, each commercial data analysis software package allows at least a few types of analysis to be performed. These software packages are either sold bundled with an image acquisition instrument (and usually limited to data produced by that company's instruments), or they are sold separately: for example, SpotFire (http://www.spotfire.com), CellMine (http://www.bioimagene.com) and GeneData Screener (www.genedata.com). The main limitation in existing software packages is often the lack of ability to

explore and analyze per-cell data and/or to link data points to images (AcuityXpress from Molecular Devices being an exception, http://www.moleculardevices.com/pages/software/acuityxpress.html). We hope that the future release of the open-source CellVisualizer software package will address many of the current data exploration demands.

Next steps

Identifying chemicals that yield positive results in the primary screening assay is only the first step, of course. Confirmation screens and secondary follow-up assays are required to lend confidence that the compounds truly have the desired effects on cells. Furthermore, with cell-based screens, identifying the target proteins of interesting chemical compounds remains no simple task, though many technologies are being developed[31,47]. In fact, one promising new approach made feasible by image-based screening is to simultaneously screen an RNA interference library to identify which genes, when perturbed, produce the phenotype sought from the chemical library[32]. Another use of RNA interference or other genetic screens is to search for genetic perturbations that are able to suppress or enhance the chemical compound's effects (modifier screens)[48].

We are only beginning to see the dramatic advances made possible by the first generations of image-based screening technologies. Cutting-edge developments in this field promise to provide even higher-content information, for example by allowing collection and analysis of time-lapse images from live cells of each sample, thereby revealing the kinetics of biological processes, or three-dimensional images of each cell sample, for better structural resolution[22]. As has been the case since the launch of this field, new biological techniques and tools, advanced instrumentation and increasingly sophisticated and easy-to-use software tools continue to drive forward the cutting edge of image-based chemical screens.

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The author declares no competing financial interests.

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