Acupuncture and Herbal Medicine

Guidelines for application of high-content screening in traditional Chinese medicine: concept, equipment, and troubleshooting

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Abstract

High-content screening (HCS) technology combines automated high-speed imaging hardware and single-cell quantitative analysis. It can greatly accelerate data acquisition in cellular fluorescence imaging and is a powerful research technique in traditional Chinese medicine (TCM). An increasing number of laboratories and platforms, including TCM laboratories, have begun utilizing HCS systems. However, this technology is still in its infancy in TCM research and there is a lack of sufficient experience with the associated concepts, instrument configurations, and analysis methods. To improve the understanding of HCS among researchers in the field of TCM, this paper summarizes the concept of HCS, software and hardware configuration, the overall research process, as well as common problems and related solutions of HCS in TCM research based on our team's previous research experience, providing several research examples and an outlook on future perspectives, aiming to provide a technical guide for HCS in TCM research.

Keywords: High-content imaging, High-content screening, Traditional Chinese medicine

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Background

High-content screening (HCS)[1] uses an automated microscope to perform multi-parameter imaging, provides quantitative data of cell populations in a high-throughput format, and saves considerable manpower and time in large-scale applications such as drug screening and systems biology for biomedical research. The general process of HCS includes disease modeling, biological sample acquisition, fluorescent labeling of certain targets associated with the disease of interest, image acquisition with an automated HCS system, final image analysis, and data statistics to obtain useful biological insights.

HCS provides a large amount of cellular information and can be used to determine information that is difficult to acquire using conventional microplate reader-based biochemical detection methods, such as single-cell information, live-cell changes, and localization of the measured substances. HCS has developed rapidly since it was proposed, and the current commercial HCS hardware and software-integrated system has become a popular basic instrument among pharmaceutical companies and has also been purchased by an increasing number of laboratories to accelerate biomedical research.

HCS has good application prospects in traditional Chinese medicine (TCM) research. TCM theories on diagnosis and treatment are available; however, explaining Chinese medical theories with modern science is difficult in modernization of TCM. The use of biological phenotypes to explain the theories of *qi* and blood, cold, and heat in Chinese medical theories[2] has been accepted to a certain extent. Phenotypic changes are visible macroscopic alterations resulting from the joint action of various targets and pathways. The detection of phenotypes is consistent with the systematic and holistic views of Chinese medical theories, which are expected to provide the scientific connotations of TCM theory in understanding and treating diseases. With the continuous advancement of new probes and other labeling technologies, it is possible to simultaneously obtain phenotypes of up to seven differently colored probes through HCS[3], which

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will help explain Chinese medicinal theories. Chinese medicines have complex compositions and functions; they often contain various compounds, and the targets and pathways of each compound differ, forming a complex network of components, targets, and pathways[4]. For pharmacological and toxicological studies of Chinese medicines, the complex multi-component and multi-target characteristics have led to a demand for high-throughput detection. HCS has high throughput and can help dissect the effects of complex Chinese medicines. In the future, drug synergy research will be performed for both drug discovery and TCM combination theory. The throughput demand for the detection of drug combinations has been reported to be considerably higher than that for the detection of single drugs[5]. In summary, Chinese medical theories have system and holism characteristics, whereas Chinese herbal medicines have multi-component and multi-target characteristics. HCS technology can be used to achieve high-throughput phenotypic analyses, which is useful for conducting experimental research in line with the characteristics of TCM.

The use of HCS in TCM research is increasing. Like ordinary and confocal fluorescence microscopes, the HCS system has gradually become popular in TCM laboratories, and many individuals involved in TCM carry out HCS experiments. However, TCM research based on HCS is still in its infancy, and there is a lack of sufficient experience in the related concepts, software and hardware configurations, experimental process design, and troubleshooting. To strengthen the understanding of TCM research related to characteristics, advantages, and procedures of HCS, as well as to promote the dual use of HCS and TCM, this paper first briefly introduces the concept of HCS and the characteristics of its hardware and software to allow readers to have a preliminary understanding of its advantages. Next, the application process of HCS in research is elucidated. Further, based on our previous research experience, common problems and solutions in HCS practice are provided as a guide for researchers. Lastly, specific TCM research examples based on HCS are provided in the context of disease target identification, drug discovery, drug safety control, and drug quality control. The growing trends in HCS in the future are also postulated.

Brief introduction of HCS: concept, hardware, and software

The HCS system consists of microscopic imaging hardware and image analysis software (Figure 1); it can be understood as a set of specialized microscopic equipment. Its characteristics and advantages are reflected in the high-speed automatic acquisition of multi-channel, multi-well, multi-site, and multi-layer images. It is equipped with software that can analyze images in batches to obtain single-cell, single-site, and single-well data.

Hardware

The hardware of the HCS system includes a light source, filter, objective lens, and camera. In particular, the HCS system is equipped with a motorized stage and autofocus

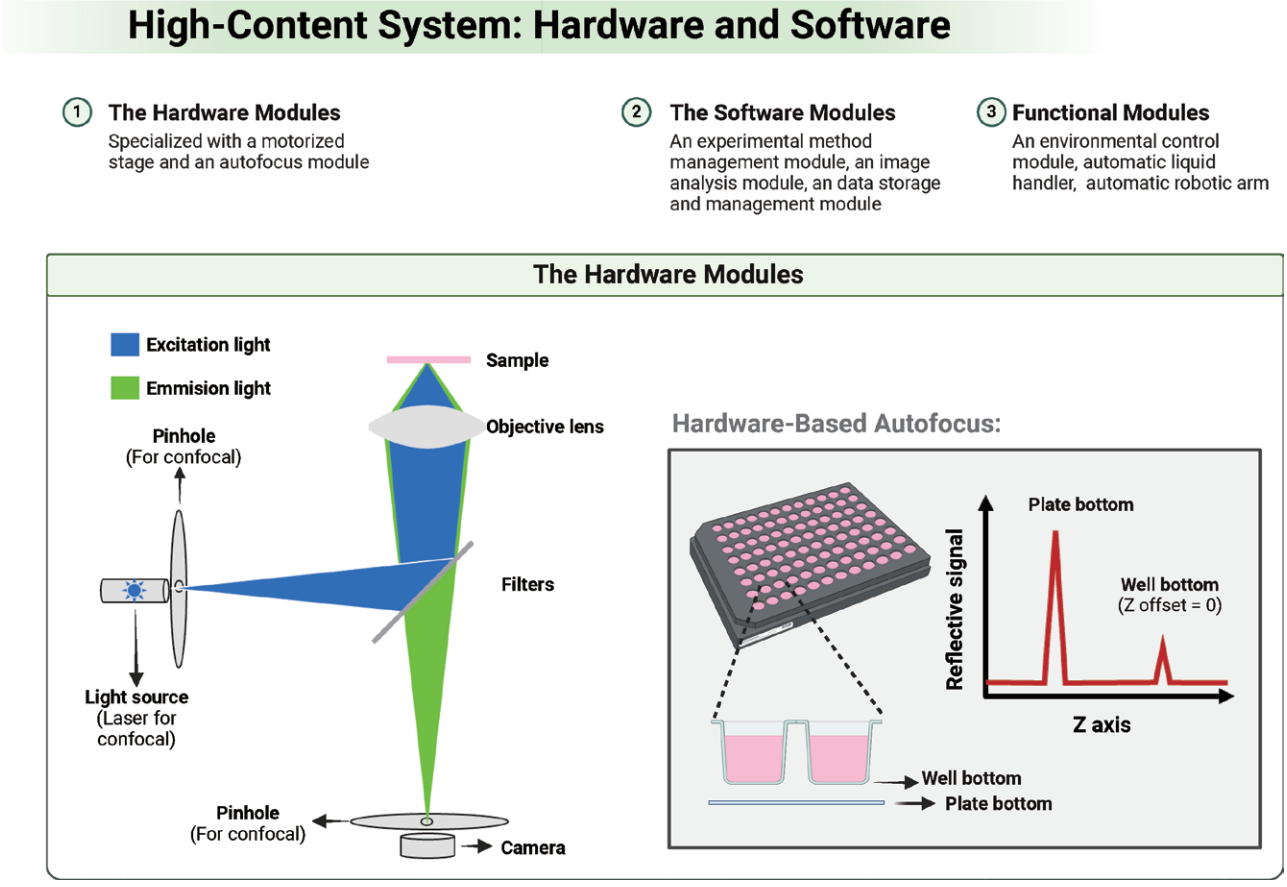

Figure 1. The composition of the high-content screening system.

module, enabling high-speed automated image acquisition. The electric stage can be accurately moved to a certain position in the XY direction, avoiding errors in the manual selection of the sites. The autofocus function consists of hardware- and software-based autofocus. Hardware-based autofocusing uses an infrared beam to illuminate the subject and measures the Z-axis light reflection signal, allowing the instrument to identify the Z-axis position of the well and plate bottoms. Software-based autofocus identifies the image with the highest quality, that is, the image with the largest signal or strongest image sharpness[6]. Consequently, the autofocus module enables the HCS system to automatically recognize the focal plane and reduces time needed for manual focusing.

According to the imaging mode, HCS systems can be categorized into widefield and confocal imaging systems. The ImageXpress Pico Automated Cell Imaging System (Pico), ImageXpress Nano Automated Imaging System (Nano), ImageXpress, Molecular Devices Micro (Micro), and ThermoFisher's CellInsight CX5 High Content Screening Platform (CX5) are wide-field HCS systems. The wide-field imaging mode is sufficient for capturing images of adherent cells. However, for thick samples such as three-dimensional (3D) spheroids or organoids, laser confocal imaging with a laser light source and confocal module is suitable. The laser beam can be focused on a small spot and can provide much higher intensity than halogen or light emitting diode (LED) lamps[7]. When the laser light source is paired with a confocal pinhole blocking stray light contamination from the nonfocal plane of thick samples, the images become of higher quality[8]. The ImageXpress Confocal HT.ai High-Content Imaging System (HT.ai) and ImageXpress Micro Confocal High-Content Imaging System (IXM-C) from Molecular Devices (San Jose, USA), and the Opera Phenix High-Content Screening System (Phenix Plus) from PerkinElmer (Shelton, USA) are representative confocal HCS systems.

Software

The software components include an experimental method management module, image analysis module, and data storage and management module. Through the experimental method management module, all conditions set to complete an image acquisition task can be stored and recalled. Communication between the image analysis module and the data storage and management module solves the major problem of analyzing a large number of images of multiple wells and sites, and can export image features at the single-cell, single-site, and single-well levels without any programming knowledge.

The image analysis module includes many built-in applications, custom module editors (CME), and artificial intelligence (AI) software. Built-in applications encapsulate a set of conventional computer vision algorithms for analyzing routine biological processes. For example, MetaXpress presents a range of built-in image analysis applications, including angiogenesis, tube formation, cell cycle, cell proliferation, cell health, cell scoring, multi-wavelength cell scoring, nuclei counting, granularity, live/dead counting, micronuclei identification, mitotic index, monopole detection, Transfluor assay, neurite outgrowth, and translocation.

If built-in applications are unable to satisfy the image analysis prerequisites, the CME can be used for more personalized solutions. The workflow of the CME is shown in Figure 2. After using multiple staining techniques to acquire the original image, a mask is generated based on the staining of distinct cell structures. By applying various threshold techniques, users can adjust the mask to achieve accurate object identification and obtain the desired outcome.

If the results do not meet the desired standards, AI software (such as IN Carta) can be utilized to achieve the customized requirements. Deep learning segmentation, machine learning classification, and 3D segmentation measurements, as well as viewing are included in the AI module. AI-powered solutions simplify the segmentation process by leveraging user annotations to train a reliable segmentation model. It also simplifies the process of stratifying cell populations, leading to an improved ability to distinguish and identify subtle, yet relevant, phenotypic differences. Importantly, AI software in HCS offers a user-friendly interface that enables biological

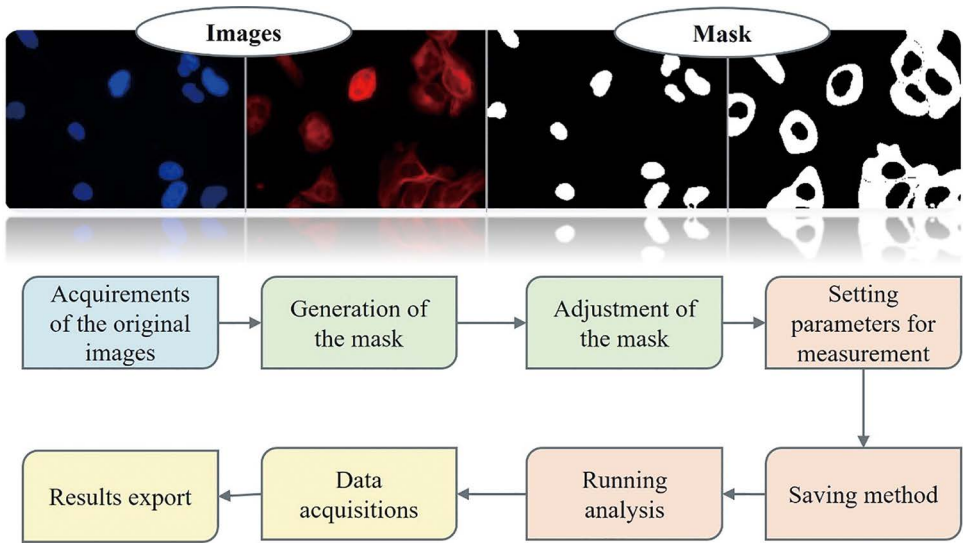

Figure 2. The workflow of a custom module editor (CME).

researchers without programming knowledge to take advantage of AI in image analysis.

Functional modules for specific applications

In addition to the basic modules described above, the HCS system can be loaded with other functional modules, such as an environmental control module, automatic liquid handler, and automatic robotic arm device, to suit special scenarios such as live-cell observation, rapid kinetic experiments, and automatic integration. (1) The environmental control module can control CO_2 and O_2 concentrations, temperature, and humidity to ensure that living cells are maintained in a good state during long-term monitoring. (2) Rapid kinetic experiments, such as calcium flux detection, require image acquisition within a very short period after sample processing[9]; therefore, it is necessary to use an automated liquid handler to add liquid without moving the plate or dish and simultaneously acquire images. Some HCS systems can be refitted with an automated liquid handler, such as the Opera Phenix® Plus high-content screening system. (3) The robotic arm unit takes samples in or out of the HCS system, thus integrating the system with other automated devices (automatic microplate washers, liquid-handling workstations, and automatic incubators) for unmanned operations.

HCS workflow in TCM research

As mentioned previously, the automatic stage, autofocus module, and batch image analysis software provide the HCS system with significant advantages for high-speed automatic image acquisition and analysis. Therefore, it provides powerful tools and technical means for studying the efficacy and toxicity of Chinese medicines as well as the mechanisms of Chinese medical theories. These include the search for molecular markers of TCM patterns from clinical samples, evaluation of the safety and effectiveness of Chinese medicines, study of the material basis and mechanism of action, and understanding of the scientific basis of Chinese medical theories, such as the four *qi* and five flavors.

The basic research flow is illustrated in Figure 3. First, a disease model is constructed or clinical samples are obtained from patients. Second, the specific targets to be tested are fluorescently labeled. Third, multiple images of the labeled samples are automatically collected using the HCS system. Lastly, the images are analyzed in batches. In addition, data analysis runs through all aspects, including experimental quality control during methodological research, data standardization after batch experiments, and hit selection.

Sample acquisition

Obtaining samples from patients diagnosed with a certain pattern (syndrome) in TCM or developing animal models of human diseases, followed by obtaining biospecimens, is the first step in analyzing the principles of disease prevention and treatment in Chinese medical theories with HCS. Patients, animals, or *in vitro* models have been treated with Chinese herbal medicines in

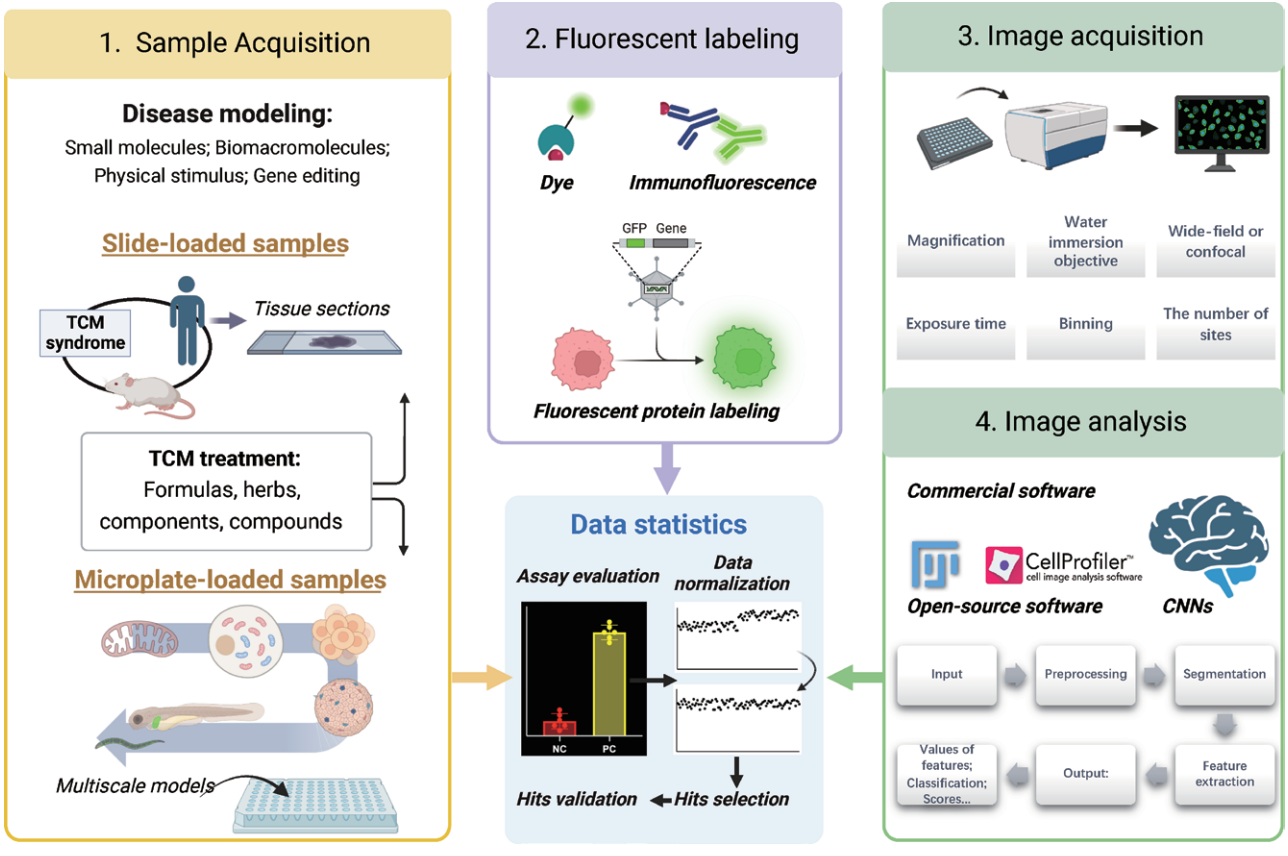

Figure 3. Flowchart of HCS in the research of TCM. CNNs: Convolutional neural networks; HCS: High-content screening; NC: Negative control; PC: Positive control; TCM: Traditional Chinese medicine.

different forms, including formulas (formulas consisting of multiple herbs), herbs, components (components of different polarities that are separated from the extracts of formulas or herbs), or pure compounds.

The methods of disease simulation (disease modeling) often include: (1) applying small-molecule compounds, such as treatment of cardiomyocytes with phenylephrine and administration of isoproterenol in mice to induce myocardial hypertrophy; (2) the application of biological macromolecules, such as recombinant transforming growth factor-β (TGF-β) treatment to fibroblasts to induce fibrosis; (3) physical stimuli, such as cultivating cardiomyocytes in a hypoxic environment and ligating the left anterior descending coronary artery in mice to simulate myocardial ischemia; (4) gene editing technology, such as using ApoE knockout mice to simulate hyperlipidemia.

Ensuring that samples from disease models are compatible with HCS systems is a prerequisite. The HCS system is compatible with both microplates and tissue sections. Two-dimensional (2D) cultured cells, 3D cultured spheroids and organoids, *Caenorhabditis elegans*, and zebrafish embryos can all be placed in 96-well plates, allowing high-throughput and high-content imaging and analysis. When combined with a slide holder, images of animal tissue sections can be captured automatically. Therefore, the HCS system is compatible with the detection of multi-scale models, including cell cultures and small or large animals. Among them, 2D cultured cells are still the most commonly used experimental tools for HCS, including primary cells, immortalized cell lines, and stem cell-derived cells. Owing to advances in biomaterials, stem cell differentiation technology, and imaging technology, 3D cultured cells are becoming increasingly popular. Commonly used 3D cells include spheroids without scaffolds, scaffold-based spheroids, organs-on-a-chip, and 3D cell cultures with different shapes obtained *via* 3D bioprinting[10]. Among them, spheroids can be produced with high throughput and good stability, and have good compatibility with HCS systems. Currently, some organs-on-a-chip have been designed to be compatible with HCS instruments, such as MIMETAS's organoid in 96/384-well plates and Emulate's organoid-on-a-chip. *Caenorhabditis elegans* and zebrafish are easy to breed, genetically manipulate, and fluorescently label, allowing HCS application in the context of a whole animal.

Fluorescent labeling

The labeling of specific targets of interest (nucleic acids, proteins, ions, organelles, etc) in cells is the second step in TCM research using HCS. Fluorescent labeling is the process by which a fluorescent probe reacts or interacts with a specific target so that the target can be visualized by fluorescence imaging.

Fluorescent labeling can be achieved using the following methods: (1) Adding a small-molecule fluorescent probe that reacts with or interacts with the target through its specific chemical structure. Taking MitoSOX™ Red mitochondrial superoxide indicator as an example, its dihydroethidium moiety forms 2-hydroxyethidium when oxidized by superoxide, and exhibits red fluorescence[11]. A variety of small-molecule fluorescent probes have been designed to specifically label various targets, including specific cell components, metabolites, metal ions, and hydrogen ions; (2) Specifically labeling the target *via* an antigen-antibody reaction. Immunofluorescence is a representative approach for this purpose. Immunofluorescence offers high specificity; however, owing to the large molecular weight and poor permeability of antibodies, the sample must be fixed and permeabilized, making immunofluorescence unsuitable for live-cell imaging. (3) Specifically labeling the target using the complementary base pairing rule. A representative approach is fluorescence *in situ* hybridization, which uses fluorescently labeled nucleic acid probes to visualize target DNA sequences or mRNA transcripts in cells. (4) Using genetic engineering techniques to transfect cells with specially designed plasmids to express fluorescent proteins (FPs). Owing to the flexibility of genetic engineering, fluorescent labeling methods have been greatly enriched since the discovery of FPs, as represented by jellyfish green fluorescent protein (GFP). Further details of fluorescent labeling with genetic engineering are discussed in the next paragraph.

Genetic engineering technology to enable cells to express FPs has various applications. (1) Using FP as a reporter gene to detect the transcriptional activity of the promoter of interest. This was one of the earliest applications of FPs. When constructing the plasmid, the FP gene sequence is placed downstream of the promoter of interest. The level of FP expression in the plasmid-transfected cells can be detected using fluorescence microscopy or a microplate reader to report the transcriptional activity of the promoter. (2) Overexpression of fusion proteins to visualize target protein localization. This is one of the most common applications of FPs. The FP sequence is added before or after the target gene, and the transfected cells express the fusion protein of the target gene and FP, which allows researchers to visualize protein localization and migration or to label certain organelles. For example, overexpression of GFP-53BP1 (focus forming region) fusion protein helps in the visualization of 53BP1 recruitment to form DNA damage foci. Overexpression of the GFP-CD9 fusion protein is useful in labeling exosomes rich in the membrane protein CD9. Additionally, based on fusion protein overexpression techniques, fluorescence resonance energy transfer (FRET) can be used to study protein interactions[12] under physiological conditions in living cells. (3) Labeling the endogenous target protein using CRISPR/Cas9 to visualize its localization. Overexpression of the fusion protein described above exogenously introduces the target protein, which may influence the endogenous stoichiometric equilibrium of the protein of interest and lead to ectopic cell localization of the protein of interest, or even abnormal cellular phenotypes. To avoid artifacts caused by overexpression and to give the tagged protein a more native level, CRISPR/Cas9 genome editing technology has been used to add the DNA sequence of a FP to the endogenous DNA sequence of the protein of interest, thereby visualizing the localization kinetics of the endogenous protein under endogenous regulation[13,14]. Research aimed at reducing off-target effects will further promote the application of fluorescent labeling using CRISPR/Cas9. (4) Construction of genetically encoded fluorescent

Table 1
Selection of imaging parameters and the associated advantages and disadvantages

Imaging parameters	Advantages	Disadvantages
A higher lens magnification	Higher resolution	Longer autofocus time is required, more sites per well are needed to capture enough cells, and thinner plates are needed because of the shorter working distance of the objective
Water immersion objective instead of air objective	Higher signal	Longer running time is needed
Confocal imaging mode instead of wide-field imaging mode	Higher resolution; 3D images of thick samples can be captured	A stronger light source or longer exposure time is required
A longer exposure time	Brighter images	Increased likelihood of quenching and phototoxicity
A higher pixel binning	Higher signal	Lower resolution
More sites per well	More cells can be captured	Longer running time

3D: Three-dimensional.

biosensors (GEFBs) to detect specific physical and chemical signals. A GEFB consists of a sensing domain and a FP; when the signal molecules bind to the sensing domain, the conformation of the GEFB changes, leading to a change in the fluorescent properties so that the signal can be converted to fluorescence changes. The sensing domain can be derived from a protein sensitive to specific signal transduction events, including cell surface receptors, intracellular messengers, and enzymes such as calmodulin, which is sensitive to calcium concentration[15,16]. For example, voltage sensitive fluorescent protein-Clover mRuby2 (VSFP-CR) is a voltage detector consisting of a voltage-sensing transmembrane protein and a GFP/RFP FRET pair that fluoresces according to voltage changes in cardiomyocytes[17].

Image acquisition

After labeling the targets in the samples, the labeled samples are placed in an HCS system for high-speed automated image acquisition. The image acquisition process involves the setting and adjustment of six main imaging parameters: lens magnification, objective lens type, imaging mode, exposure time, pixel binning, and the number of sites per well. Selection of imaging parameters and the associated advantages and disadvantages are listed in Table 1.

Image analysis

Commonly used image analysis solutions include commercial software, open-source software, and open-source codes. Commercial software, such as MetaXpress (Molecular Devices) and Harmony (PerkinElmer), and open-source software, such as ImageJ and CellProfiler, provide packaged image analysis modules that can directly output values of image features, such as morphology, number, and intensity. Compared with packaged software, open-source code is more flexible, and there are many open-source deep learning platforms, including Caffe, TensorFlow, PyTorch/Caffe2, and Keras, that users can easily deploy in deep learning models.

The general image analysis process consists of four steps: image preprocessing, image segmentation, feature description, and result outputting[18].

First, image preprocessing helps increase the accuracy of the subsequent quantitative analysis. Common image preprocessing includes image resizing, image compressing, image enhancement, and shading corrections. Occasionally, the image needs to be resized because some machine learning methods require the input image to be uniform in size. Compressing images can reduce computing power. Application scenarios for image enhancement include improving image clarity, enhancing contrast to enhance the detailed visibility, and eliminating noise. Owing to the heterogeneity of illumination, dark corners can be observed in the microscope image, requiring shading corrections.

Second, image segmentation is the division of an entire image into multiple sections, which is an indispensable basis for almost all tasks, including nucleus recognition, cell boundary recognition, organelle boundary recognition, and spot recognition. Traditional methods of image

segmentation include threshold-based segmentation, edge detection, region-based segmentation, and clustering (including k-means clustering)[19,20]. (1) Threshold-based segmentation is the simplest and most commonly used method in fluorescence image segmentation. It divides the image into two parts: the region of interest (ROI) and the background, based on the grayscale value of pixels, and is sensitive to noise and only suitable when the ROI and background have a high difference in brightness. Commonly used thresholding methods such as Ostu, Intermodes, MinError, and Percentile are embedded in ImageJ. (2) Edge detection can immediately detect edges using pixel values. Its advantage is that it does not necessarily require closed borders[21], and its disadvantage is that it is not suitable for pictures with too many edges. (3) Region-based segmentation relies on seed selection and neighbor growth. Its advantage is that it correctly follows homogeneity criteria when defining a region, whereas its disadvantage is that it requires considerable time and memory. (4) In k-means clustering, similar pixels are grouped into clusters. The algorithm is simple; however, it is sometimes difficult to determine the number of clusters, that is, the *k* value. In addition to traditional methods, deep learning has also recently been used in image analysis. Deep learning-based approaches can be used to solve complicated image segmentation tasks, even with significant interference (eg, light intensity changes, noise, and complex backgrounds). Deep learning-based approaches exhibit excellent performance compared to traditional image segmentation methods in many segmentation tasks[22]. The disadvantage of deep learning-based approaches is the high demand for computing power, especially during training, which can be partially compensated by using public models that have been pre-trained with large datasets.

After obtaining the ROIs, the next step is to describe the image features within them. Based on different experiments, commonly used image features include brightness, area, shape, and diameter. For example, in experiments based on dichlorodihydrofluorescein diacetate (DCFH-DA) probes for reactive oxygen species level detection, the fluorescence brightness within each cell is a feature of interest. For more complex experiments, such as mitochondrial morphological analysis, it may be necessary to acquire dozens to hundreds of morphological features, ranging from natural features that directly quantify mitochondrial geometry to calculated features such as the average and standard deviation of all mitochondrial parameters of a single cell[23]. Through feature extraction or selection, multiple features can be combined into fewer features, or more representative features can be selected to reduce the difficulty of subsequent computation. Common feature extraction methods include principal component analysis (PCA), independent component analysis (ICA), and linear discriminant analysis (LDA)[24]. With the development of deep learning, convolutional neural networks (CNNs) can be used to automatically extract features. Pre-trained open-source CNN architectures such as VGG, ResNet, and DenseNet are often used as feature extractors and have shown superiority in various fields of computer vision, including cell image analysis[25].

Fourth, the output result is the final judgment of the image based on the extracted image features. For simple image analysis tasks, the output is the value of one or more image features that are most representative, such as the number of cells, cell area, and fluorescence intensity. For a task involving machine learning, the output may be the classification or score of the image.

Data statistics

Before performing large-scale experiments, it is necessary to evaluate and confirm the robustness and reliability of the assay. Relying on the *t* test alone is too arbitrary. Z'-factor[26] and SSMD[27] are statistical parameters used to quantitatively assess whether an assay method can be used for high-throughput screening. These parameters are calculated as follows:

$$Z' = 1 - \frac{3\times \mathrm{std}(C_{\mathrm{pos}}) + 3\times \mathrm{std}(C_{\mathrm{neg}})}{|\mathrm{mean}(C_{\mathrm{pos}}) - \mathrm{mean}(C_{\mathrm{neg}})|} \quad (1)$$

$$\mathrm{SSMD} = \frac{\mathrm{mean}(C_{\mathrm{pos}}) + \mathrm{mean}(C_{\mathrm{neg}})}{\sqrt{\mathrm{std}(C_{\mathrm{pos}})^2 + \mathrm{std}(C_{\mathrm{neg}})^2}}, \quad (2)$$

where C_{pos} is the positive control group, C_{neg} is the negative control group, and std is the standard deviation. Taking high-content drug screening as an example, positive controls refer to known active compounds and negative controls to inactive compounds or solvent controls.

The larger the Z' factor, the better the assay method can reflect the difference between the positive and negative controls. When $0.5 \le Z' < 1$, the assay method is good. When $0 < Z' < 0.5$, the assay is acceptable; when $Z' < 0$, the assay is unacceptable. If the absolute value of SSMD is ≥3, the assay has passed the SSMD-based quality inspection; if SSMD <3, the assay has failed the quality inspection.

Once data from large-scale experiments are obtained, they must be normalized to correct for the differences between plates. There are two main methods for normalizing data: control-based and non-control-based. The control-based method uses the positive control or the negative control on the same plate as 100%, corresponding to the percentage of control (PC) formula. However, positive and negative control wells are also subject to variability; therefore, a non-control-based normalization method is preferred in most cases, that is, the mean or median of all tested wells in the same plate is used as 100%, corresponding to the percentage of samples (PS) and the robust percentage of samples (RPS) formulas[28] as follows:

$$\mathrm{PC} = \frac{S_i}{\mathrm{mean}(C)} \times 100 \quad (3)$$

$$\mathrm{PC} = \frac{S_i}{\mathrm{mean}(C)} \times 100 \quad (4)$$

$$\mathrm{RPS} = \frac{S_i}{\mathrm{median}(S_{\mathrm{all}})} \times 100, \quad (5)$$

where S_i is the sample group i, C is the control group, and S_{all} are all the sample groups.

For the application of HCS in drug screening, after the screening data is normalized, Chinese medicines with a value higher or lower than that of the control group/model group are determined as hits, and further validation and related studies are performed. To avoid false positives, it is necessary to choose a Chinese

medicine that is sufficiently different from the control or model groups. The methods for selecting the hits include the following: (1) Setting a percentage cutoff, such as selecting Chinese medicines with an inhibition rate higher than a certain percentage or directly selecting ones with the highest efficacy. (2) Selecting Chinese medicines with a value higher or lower than mean ± 3 × std. (3) Selecting Chinese medicines with a value higher or lower than mean ± 3 × median absolute deviation (MAD). (4) Identifying hits using Bayesian statistics[29].

Finally, the hits are validated using the following methods: Retesting the hits and using statistical tests such as the *t* test to determine statistical differences between the hit group and the control/model group[28]. Additionally, the hits should have dose-dependent effects. Cells are treated with different concentrations of hits, and the EC_{50} (the concentration at 50% effective rate) values are calculated.

Common problems and troubleshooting

For high-content imaging and analysis, the selection of the sample preparation method, imaging parameters, and image analysis method can influence the final experimental results. With an inappropriate selection, serious problems can arise, such as large batch-to-batch variations, poor image quality, and inaccurate image analysis. To improve the quality of high-content imaging and analysis, researchers have attempted to determine the cause of these problems and resolve them.

Reducing batch-to-batch sample variation

Increasing cell quality

Cells overexpressing FPs gradually lose FP expression as the number of passages increases[30]. Too many passages should be avoided.

Microbial contamination can compromise the results of the cell experiment[31]. Prevention recommendations have been previously reviewed in detail[32]. It is worth noting the Chinese medicines under investigation can be a source of contamination; thus, if water is used as the stock solvent, it is necessary to sterilize the solution through a 0.22 μm filter before treating cells.

Ensuring reagent consistency

Freeze-thaw cycling, light exposure, solubility, and temperature are the four main factors that may influence the characteristics of reagents and decrease batch-to-batch consistency. (1) For some modeling reagents and Chinese medicines, the freeze-thaw process[33] causes structural changes to the constituent compounds. To ensure stability, it is recommended to divide the stock solution to avoid freeze-thaw cycling. (2) Reagents with poor photostability must be protected from light during processing. (3) When diluting the stock solution of Chinese medicines into a working solution, it is necessary to fully ultrasonicate, vortex, or add a co-solvent or suspending agent to fully dissolve or suspend the herb to ensure that the concentration of the working solution is consistent. (4) Temperature is a factor that has a profound impact on the brightness of staining, but is often overlooked. It is important to precool/preheat the reagents, including the staining working solution and washing buffer, to their working temperature in advance to ensure staining stability.

Increasing the quality of captured images

Selecting appropriate microplates

A black-walled microplate with a thin and optically clear bottom offers low well-to-well interference and background fluorescence. Microplates of appropriate quality include Corning #3603/#3904, #4580, and #4581, Cyclo-Olefin Copolymer (COC) series, PerkinElmer ViewPlate series, CellCarrier series, Greiner Bio-One #655090, CELLview series, and SensoPlate series.

Optimizing imaging parameter selection

The selection of imaging parameters markedly affects the signal-to-noise ratio (SNR) of the image. Higher lens magnification, confocal mode, and smaller confocal pinholes all result in higher resolution, but significantly reduce the fluorescent signal.

Switching to water immersion objectives, increasing the intensity of the light source, and increasing the exposure time are possible solutions for increasing the SNR.

Optimizing autofocus settings

Different autofocus parameters are set for different plates. Although the autofocus is set by the engineer, checking and perfecting the autofocus settings before each experiment will better avoid out-of-focus related to differences between plates. During autofocusing, two reflection peaks should be observed: one is that of the well bottom, and the other is of the plate bottom (Figure 1). The distance between the two peaks represents the bottom thickness. Re-measuring and updating the bottom thickness value should be performed to ensure that the HCS system goes through autofocusing within the appropriate *Z* range, and signals from both peaks are always received.

Improving image analysis accuracy

Adding image preprocessing steps

Image preprocessing is performed on the original image before quantification so that noise can be removed or useful information can be restored or enhanced. (1) Denoising. Noise is unavoidable in fluorescence microscopy images because of photons loss and measurement noise[34]. Many microscopy institutions have developed a deconvolution toolkit to correct image blur using computational deconvolution methods such as deblurring, nearest neighbor, and inverse filter[35]. (2) Contrast enhancement. Contrast enhancement uses the difference in the brightness of the pixels within an image to make the details more obvious. Both histogram stretching

and equalization can enhance the image contrast. (3) Deep learning-based image preprocessing. Deep learning provides effective support for fluorescence microscopy image preprocessing; for example, in 2021, Hu et al. proposed the use of SRACNet to enhance image details, reduce noise, suppress artifacts, and correct aberrations[36].

Choosing appropriate image segmentation methods

In most cases, it is necessary to segment the ROI where the cells/analytes are located. For images with a low SNR, where the commonly used threshold-based method does not meet the requirement, other segmentation methods can be tested, among which the deep learning-based methods CellPose[37], U-Net[38], and the Segment Anything Model[39] are well-known accessible tools. For example, CellPose 1.0 is available on-line (http://www.cellpose.org), and CellPose 2.0 provides a graphical user interface for users without programming knowledge to train their own models.

Research examples

From the discovery of disease targets to the construction of multi-scale disease models, the discovery of effective drugs from Chinese medicines, and finally, the quality and safety control of Chinese medicines, HCS has a wide range of research applications in all aspects of TCM research (Figure 4).

Studying disease pathology and potential therapeutic targets

The observation of clinical samples using high-content imaging is expected to provide new evidence for the study of disease pathology. For example, Chen et al.[40] collected gastric mucosal biopsies from patients with chronic atrophic gastritis and healthy individuals. They observed microRNA-7 downregulation in lesions, which was restored by the Chinese medicine Yiwei Xiaoyu granules. The research results provide new experimental evidence for potential therapeutic targets in the gastric mucosa and uncover the potential mechanism of action of Chinese medicines[40].

Construction of multi-scale disease models

Researchers have constructed a variety of disease models compatible with HCS at different scales, such as molecular, subcellular, cellular, organoid, and animal levels (Figure 5).

For example, at the molecular level, Jiang et al.[41] developed a specific fluorescent probe to image superoxide molecules in cells, applied it in HCS of modulators of oxidative stress, and identified coprostanone as a modulator of superoxide homeostasis that effectively ameliorated myocardial ischemia/reperfusion injury in mice[41]. Li et al.[42] designed a fluorescent sensing system for intracellular imaging of the biomolecule histone deacetylase 1 (HDAC1) and applied molecular imaging for HCS of HDAC1 inhibitors.

At the subcellular level, our team built an irradiation-induced DNA damage model, and used an HCS system to capture the intranuclear DNA damage foci, of which the diameter was approximately 1 μm. The established model was used to identify several novel modulators of the DNA damage response[43].

At the cellular level, Guo et al.[44] established a fluorescence-based cardiomyocyte hypertrophy model and identified the bioactive compounds by HCS.

Fan et al.[45] constructed a 3D cardiac organoid-based hypertrophy model and used high-content imaging to

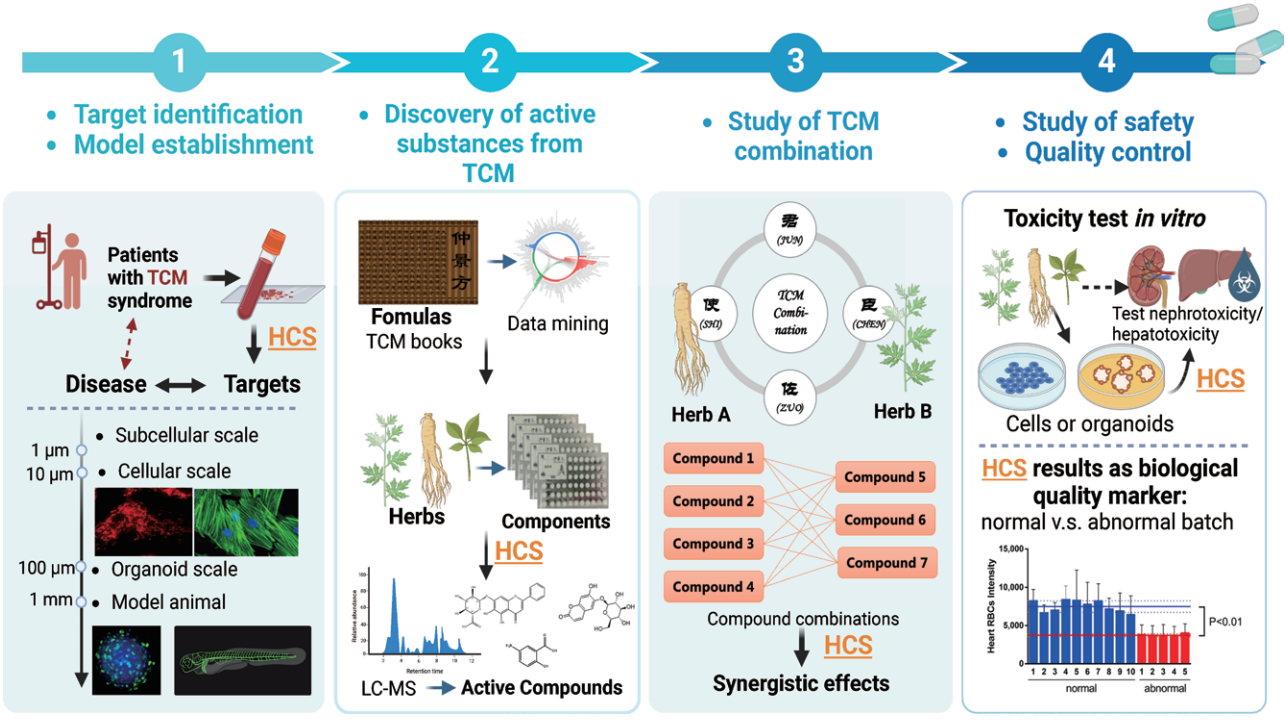

Figure 4. Applications of HCS in TCM drug research and development process. HCS: High-content screening; LC-MS: Liquid chromatography-mass spectrometry; TCM: Traditional Chinese medicine.

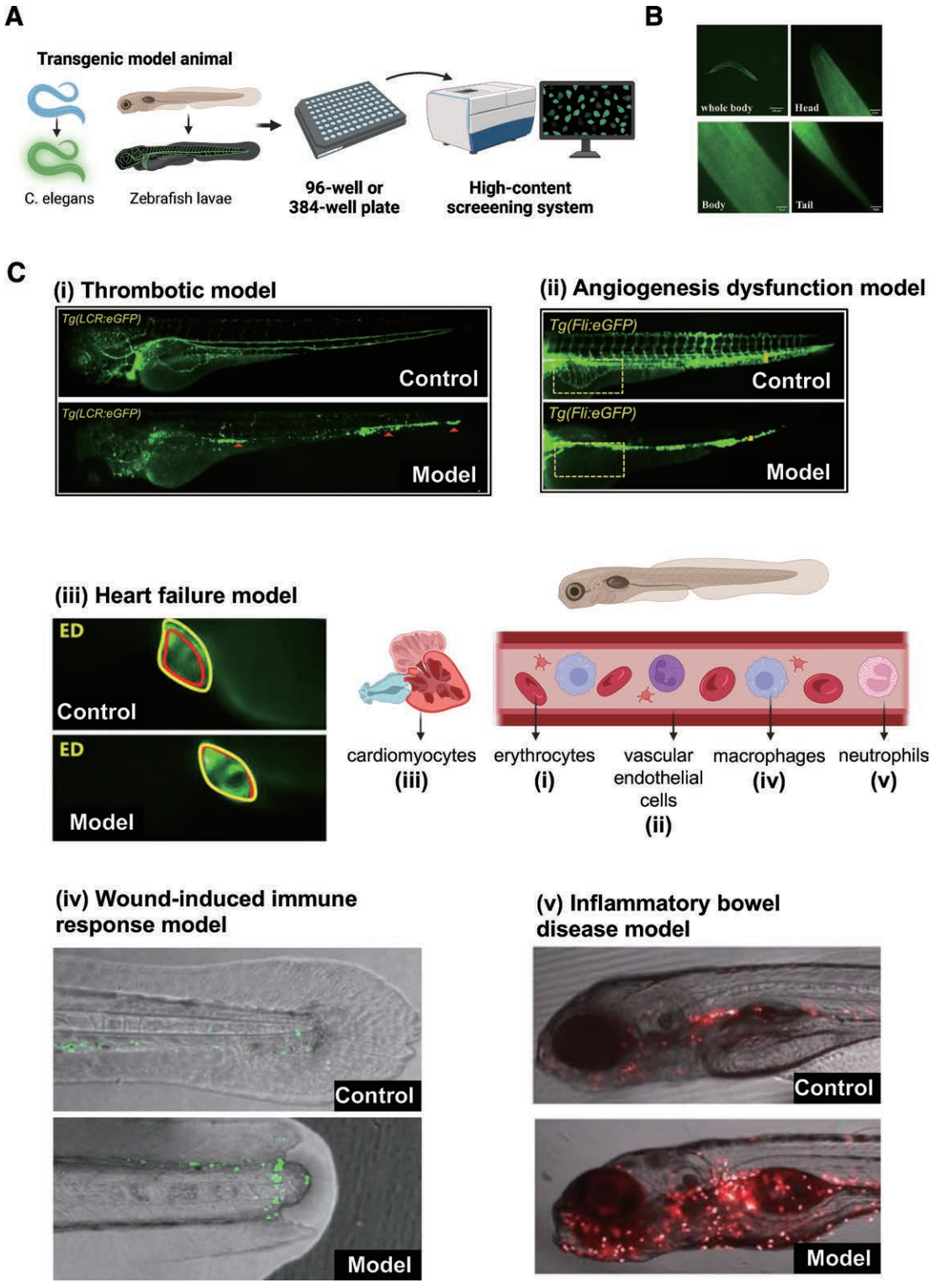

Figure 5. Integration of transgenic model animals and high-content screening. (A) The workflow of model animals-based high-content screening. (B) Epidermis collagen XII labeled *Caenorhabditis elegans*. (C) Various zebrafish disease models, including (i) thrombotic model; (ii) angiogenesis dysfunction model; (iii) heart failure model; (iv) wound-induced immune response model; (v) inflammatory bowel disease model. ED: End diastole.

study the effect of Guanxinning injection on myocardial hypertrophy and fibrosis, providing a new experimental basis for the treatment of heart failure using Chinese medicine.

Our team[46] constructed a transgenic Pcol-19-COL-12:GFP *Caenorhabditis elegans* line in which epidermal collagen XII was fluorescently labeled. An efficient HCS technique was then used to identify bioactive natural products that promote the expression of collagen XII in this model[46].

The availability of various transgenic zebrafish enables *in vivo* imaging of various zebrafish disease models. For example, our team established a thrombotic model in erythrocytes-labeled *Tg(LCR:eGFP)* transgenic zebrafish[47–49], an angiogenesis dysfunction model in vascular endothelial cell-labeled *Tg(Fli:eGFP)* zebrafish[50], a heart

failure model in cardiomyocyte-labeled *Tg(cmlc2: eGFP)* zebrafish[51,52], a wound-induced immune response model in macrophage-labeled *Tg(mpeg:eGFP)*[53,54] and neutrophil-labeled *Tg(Lyz:dsRED2)* zebrafish[54], and an inflammatory bowel disease (IBD) model in neutrophil-labeled *Tg(Lyz:DsRED2)* transgenic zebrafish[55].

In summary, in the field of HCS, the establishment of multi-scale disease models is in full swing, and their further integration with TCM research will provide a basis for disease simulation, research on Chinese medical theories, and pharmacological research on Chinese medicine.

Discovery of active substances in Chinese medicines

HCS can be used in the step-by-step identification of effective substances in Chinese medicine. For example, to identify effective formulas and compounds for the treatment of IBD, Yu et al.[56] innovatively combined knowledge mining, HCS, and high-resolution mass spectrometry to systematically screen Chinese ZhongJing formulas. First, the correlation between the 248 Zhongjing formulas and typical symptoms of IBD was analyzed. Seventy-four components were prepared from the top three formulas predicted to be associated with IBD, and their therapeutic effects were screened in a transgenic zebrafish IBD model. Six components were found to inhibit intestinal neutrophil accumulation and generation of reactive oxygen species. Subsequently, the chemical compositions of these components were analyzed by mass spectrometry, and active compounds with anti-IBD effects were clarified[55]. This study demonstrates an efficient layer-by-layer strategy for the elucidation of Chinese medicines.

Study of combination TCM

HCS has the advantage of exploring drug synergies owing to its high-throughput access to phenotypes. For example, Chen et al.[50] demonstrated the synergistic proangiogenic effects of salvianolic acid B and ferulic acid in an angiogenesis-defective zebrafish model. These results provided evidence for the combined use of two herbs, Salvia miltiorrhiza Bunge (S. miltiorrhiza) and Ligusticum striatum DC. (L. striatum), in promoting blood circulation. Li et al.[48] also used HCS to study the synergistic effects of the two herbs in regulating endogenous thrombosis and discovered that cryptotanshinone and senkyunolide I, two representative compounds derived from the herbs, had synergistic anti-thrombotic effects. The results of these studies provide biological evidence supporting the combination theory of TCM.

Safety evaluation of Chinese medicines

Due to the complex chemical properties of herbal products and the lack of appropriate evaluation methods, their safety has always been a major concern. The use of HCS is advantageous for safety evaluation of Chinese herbal medicines. For example, Wang et al.[57] used a sensitive, reproducible, multi-parametric, high-content cell-based analysis method to evaluate the liver safety of four Chinese medicine injections, which was validated by classical animal toxicity testing, and can be used for preclinical screening and post-clinical evaluation of Chinese medical preparations. Furthermore, liver organoids have been used by Liu et al.[58] for the evaluation of hepatotoxicity of Chinese medicines. They used high-content imaging of *in vitro* liver organoids to successfully distinguish the hepatotoxicity of two stereoisomers, proving the practicability of organoid-based high-content imaging methods for evaluating and predicting the organ toxicity of natural products in a low-cost and high-throughput manner.

Optimization of manufacturing and quality control of Chinese medicines

Quality control of Chinese medicines is an important issue that needs to be urgently addressed in the pharmaceutical industry. Therefore, more efficient, accurate, and scientific methods are required to improve quality control of Chinese medicines[59]. Among these, biological evaluation methods can supplement physical and chemical methods[60]. HCS, which can be used for rapid multivariate analysis of the biological activity of tested drugs, has promising applications in quality consistency evaluation. For example, Qi et al.[61] used HCS in a zebrafish thrombosis model and found that the contents of two compounds in Danhong injection, rosmarinic acid and p-coumaric acid, were positively correlated with the anti-thrombotic effect of the injection, suggesting that the two compounds could be potential quality markers of Danhong injection in ensuring batch-to-batch consistency.

Future perspectives

Integration of HCS with organoids

Although 3D culture requires a higher number of cells and is more difficult to use, it has been shown to increase cell-cell and cell-extracellular matrix interactions and is more relevant to testing *in vivo* tissues in some physiological contexts[62]. Organoids derived from stem cells are 3D cell cultures that reproduce the complex structure and function of tissues[63], allowing the study of physiological and pathological processes in an organ-mimicking environment *in vitro*. Further developments in the field of organoids, such as the introduction of immune and vascular systems, will further enhance the biological relevance of organoid models[64]. Advances in other technologies, such as organoid culturing methods, automated liquid-handling devices, and image analysis algorithms, will facilitate in improving the homogeneity and reproducibility of organoid experiments, resulting in more reliable and advanced discoveries. In addition, as organoids can be derived from patient-derived induced pluripotent stem cells or adult stem cells, they pave the way for precise and personalized medicine[65], which will provide a powerful tool for the diagnoses and treatment of patients. Simultaneously, with the gradual popularization of high-throughput organoid culture and HCS techniques, researchers are likely to obtain more scientific evidence related to Chinese medical theories, enriching the scientific implications of Chinese medicines.

Integration of HCS with microfluidics

A microfluidic device, also known as a lab-on-a-chip, manipulates the flow of liquids in a channel tens of microns in size[66]. For example, researchers have used fluid flow to induce physiological shear stress and discovered a new mechanism by which tumor cells avoid shear stress and regulate tumor cell intravasation[67]. Microfluidic technology has the advantages of miniaturization and high throughput; therefore, it has the potential to process trace samples and screen drug combinations. In addition, the structure of the cell culture space can be customized on the microfluidic chip, and biosensor devices, such as microelectrodes, can be integrated to achieve some inaccessible purposes in ordinary microplates while ensuring high throughput, such as simulating vascular barriers for studying their permeability[68], performing noninvasive electrical recording in 3D neuronal cultures[69], or coupling taste organoids with an array of extracellular potential sensors for biological taste simulation *in vitro*[70].

We previously developed a novel real-time imaging microfluidic system that integrates HCS to study TCM. In particular, independent fluid control and dynamic dosing was performed in 30 channels simultaneously, mimicking the *in vivo* dynamic concentration changes of multiple components in Chinese medicines. The schematic depicted in Figure 6 represents the developed system. The system is regulated by a computer equipped with specific software and a fluid-driving module, which facilitates precise control of the liquid volume in each channel by applying pressure. The flow-monitoring and communication modules provide feedback to the computer, which in turn adjusts the fluid-driving module. The Chinese medicine components are placed separately into distinct channels of the reservoir. Drawing on the pharmacokinetics of Chinese medicinal formulas, a real-time combination of components was simulated *in vitro* and applied to fluorescent-labeled cells. Subsequent changes in fluorescence were recorded and analyzed using HCS.

In the future, more efforts will be focused on ensuring a simpler and more versatile microfluidic platform. As microfluidic platforms become available in many laboratories, HCS based on microfluidic devices will continue to contribute significantly to TCM research.

AI-based HCS

With the rapid development of AI, image analysis has become one of its main beneficiaries. In recent years, deep learning-based AI has changed almost all image analysis tasks[71]. Advanced microscopic techniques allow us to obtain numerous still and time-lapse images; however, accurate analysis is a major challenge. Deep learning algorithms have been applied to image classification, region segmentation, and super-resolution reconstruction of microscopic images, with encouraging results[72].

In terms of image reconstruction, for example, a Deep Fourier Channel Attention Network (DFCAN) has been proposed to prove that image quality comparable to that of super-resolution microscopy can be achieved using computational methods[73]. Image reconstruction methods are expected to greatly contribute to the study of cell microstructures, which remain to be explored in most TCM studies.

In terms of region segmentation and image classification, the emergence of powerful deep learning-based image analysis methods will accelerate the automatic and accurate classification of phenotypes. For example, to segment cells and classify cellular phenotypes, our team developed a deep learning-based open-source pipeline, FociNet, which can be used to automatically segment full-field fluorescent images and classify the DNA damage status of each cell[43]. We employed FociNet on large-scale

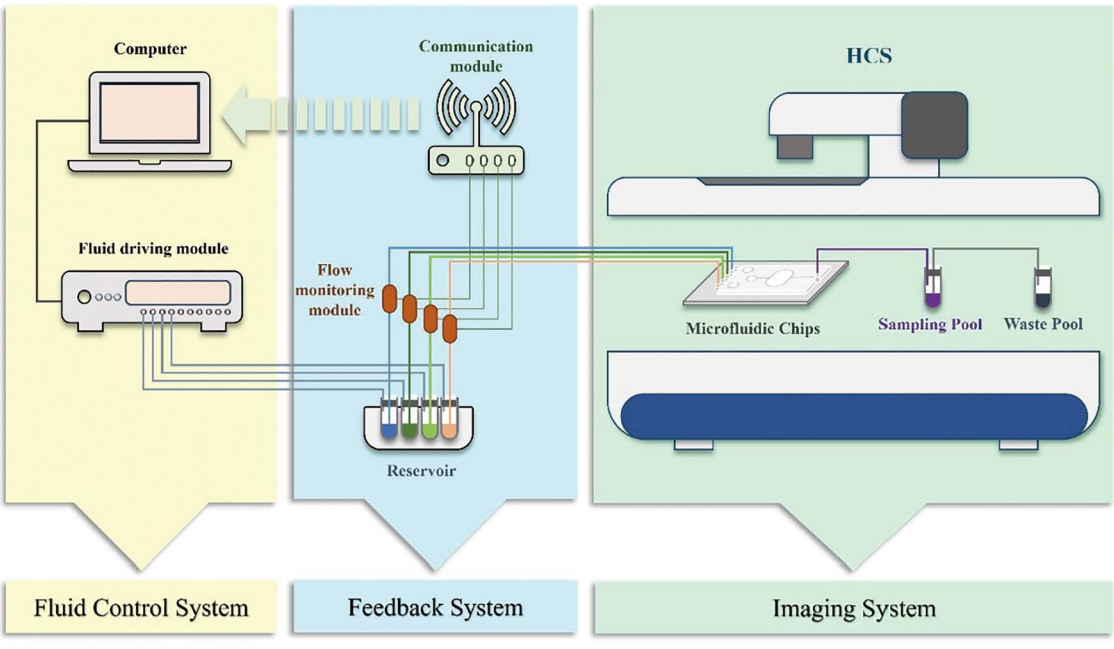

Figure 6. Schematic diagram of real-time imaging microfluidic system. HCS: High-content screening.

HCS images for the rapid and accurate identification of modulators of DNA damage response, as well as inhibitors of anthracycline-induced DNA breaks[74]. In addition, for the segmentation of model animals, Liu et al.[52] used deep learning to assess cardiac functions through motion videos of a zebrafish heart failure model, and discovered cyanidin chloride as a novel keap1 inhibitor against doxorubicin-induced cardiotoxicity. Additionally, new generalist tools for microscopic image analysis, including Cellpose[37], Scellseg[75], and Microsnoop[76], have been developed, enabling biologists to easily perform adaptive cell instance segmentation and image representation tasks.

In summary, AI-based HCS image analysis provides a rapid new approach to interpreting Chinese medical theories and identifying the mode of action of Chinese medicines.

Conclusions

The application of HCS in the field of TCM research is still in its infancy, and with the opening of new academic laboratories worldwide and the promotion of the sharing of public research platforms, HCS equipment has become available to an increasing number of researchers. With the integration of organoids, microfluidics, and AI, HCS will continue to become a powerful tool in TCM research, including research on Chinese medical theories, the mode of action of Chinese herbal medicines, and the discovery of drug candidates based on Chinese medicine. Therefore, we hope that some of the processes and problem-solving strategies discussed herein will provide a methodological guide for HCS in TCM research and encourage others to use HCS techniques to leverage their advantages in automated high-speed imaging and single-cell quantitative analysis for TCM research.

Glossaries

High-content imaging: Obtaining large amounts of image data using automated microscopes for multiparametric imaging.

HCS: Using automated microscopy to perform multi-parametric imaging and provide quantitative data on cell populations, which can be used for high-throughput screening of active drug candidates or other perturbations.

Chinese medicines: Medicines used to prevent, treat, and diagnose diseases under the guidance of Chinese medical theory.

Pattern (syndrome): A pathological summary of the disease location, etiological factors, nature, severity, and prognosis at a certain stage in Chinese medical theory.

Hit: A compound with the desired activity in drug screening.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Xuechun Chen, Mingxu Zhang, and ChunMing Lyu participated in the writing of the original draft. Lu Li, Jian Yang, and Yizhou Xu participated in the review and editing of the paper. Yang Yang and Yi Wang participated in the conceptualization.

Ethical approval of studies and informed consent

Not applicable.

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Data availability

All data generated or analyzed during this study are included in this published article.

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