Development of automatic image analysis methods for high-throughput and high-content screening

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Chapter 6

Discussion and conclusions

6.1 Developing ultra-high content analysis platforms for high-throughput screening which do not require high resolution microscopes

Over the past years, high-throughput and high-content screening has been developed and successfully applied in various screens to identify the functional role of small molecules, peptides or RNA interference (RNAi) molecules by investigating cellular phenotypic development after exposure to these agents. With advanced laboratory robotics and automated microscopy systems, high-throughput and high-content screening enables thousands of experiments to be performed simultaneously with large volumes of microscope images generated automatically. Therefore the term "high-throughput" is used. The term "high-content" refers to the information of cellular phenotypic changes and their dynamics contained in the microscope images. With an increasing interest of observing more subtle changes for more complex cellular phenotypes, several research groups have made efforts to improve microscopy techniques or implement high resolution microscopes such as spinning disc confocal microscopy [1,2] or super resolution microscopy [3,4] for the purpose of high-throughput and high-content screening, thus providing improved sensitivity and image quality and resolution. However, these technologies are often too expensive and require major technical modifications for wider availability. Therefore many laboratories cannot afford these techniques.

In this thesis, we aimed to develop robust image analysis platforms that do not require high resolution microscopes for high-throughput and high-content screening. In **chapter 2**, we investigated the NF-κB nuclear translocation dynamics based on a confocal fluorescence microscope. In order to study a sufficient number of cells from each siRNA treatment, a dry Plan Apochromat objective with relatively low magnification (20×) and low numerical aperture (0.75 NA) was used to acquire time-lapse image series. The biggest challenge was to resolve individual cells, especially in the regions where cells were touching each other or even overlap with each other. To solve this problem, we developed a novel segmentation method to estimate single cellular

area based on the topology of cells. We firstly applied watershed masked clustering (WMC) [5, 6] to detect single nuclear regions. Subsequently, the Voronoi diagram was generated to estimate the edges of cells. Finally, based on the convention that cells are ellipsoid-like objects, the best-fit ellipse in each Voronoi cell was calculated to refine single cell regions.

In **chapters 3, 4 and 5**, two images analysis platforms were developed to extract phenotype characteristics of 3D cultured micro-tissues from wide-field microscope image stacks, which contain both in-focus and out-of-focus signals due to the limited depth of field. One platform presented in **chapter 3** is based on the 2D projection. It started with compositing a single image slice by projecting only in-focus regions from each slice of image stacks. Next, watershed masked clustering was applied on the projected 2D slice to segment individual nuclei, while a local Niblack algorithm was used to define multi-cellular regions. Finally, quantification was carried out on the segmentation results and projected images, resulting in 598 parameters measured for the phenotypic profiling of each well. In **chapter 4**, we extended the 2D projection based analysis platform to enable 3D phenotypic profiling of micro-tissues. A deconvolution technique was applied to each image stack to remove out-of-focus signal by computing a point spread function (PSF) according to the optical principles. After deconvolution, we applied 3D watershed masked segmentation to detect individual nuclei. To define multi-cellular micro-tissue regions, we developed a novel segmentation method based on a sharpness level metric. By incorporating this metric in a K-means clustering method, correct intensity variation of background was estimated and a threshold was calculated dynamically for each slice. To reduce the elongated effect of nuclei in z-direction, resulting from low NA and low vertical resolution, we introduced a simple normalization method using the image of fluorescent beads with known size. Subsequently, 3D geometric models of nuclei and multi-cellular structures (also refers to as cell clusters) were reconstructed to perform phenotype quantification.

6.2 Balance between "high-content" and "high-throughput"

Image quality directly depends on several factors such as imaging modality, resolution and magnification of objective, and imaging sampling. To obtain higher quality of images, more advanced imaging techniques, higher magnification and resolution, or smaller sampling size are often required, however, this limits the throughput in high-content screening applications due to increased imaging time and computational complexity. In order to find the balance between "high-content" and "high throughput", this thesis optimized the imaging techniques, sampling sizes and image analysis modalities for different screening purposes.

6.2.1 Imaging techniques: Confocal or wide-field microscope

Compared to wide-field microscopy, confocal microscopy provides much higher resolution image stacks for 3D imaging, as each generated image slice contains only in-focus

information from the focal plane. However, the slow scanning process limits their application in high-throughput screening. Especially when the cellular structure under investigation is at a micro-tissue level, a large amount of imaging time is required to process a whole plate and consequently bleaching of the fluorescence of the specimen is caused. Therefore, wide-field microscopy is an alternative solution for the high-throughput and high-content screening of 3D cultured micro-tissues. Though the images generated by wide-field microscopes contain out-of-focus regions due to the limited depth of field, by developing an efficient image analysis method for wide-field microscope, we retrieved quantification results comparable to results generated with confocal microscope. In **chapter 4**, we developed an automated image analysis platform to reconstruct 3D micro-tissue structures from wide-field microscope images, and then profile the reconstructed structures with phenotypic parameters. We evaluated these parameters by comparing with the same parameters measured from the confocal microscope images. No significant difference was obtained, indicating that with advanced image analysis techniques, wide-field microscopy might already be sufficient for the high-content screening of 3D cultured micro-tissues to establish changes in cellular phenotypes.

6.2.2 Sampling sizes: undersampling and oversampling

For imaging, ideal sampling size is defined as the largest distance between two signal-recording points that enables to reconstruct the original continuous signal without any information loss. According to the Nyquist-Shannon sampling theorem, the conventional fluorescence wide-field microscope that was used in **chapters 4 and 5** should have the ideal axial sampling size (also referred to as pixel size) of 679 nm and vertical sampling size (also referred to as z-sampling step size) of 16882 nm. However, due to the limitation of the microscope system, which is that the smallest sampling size in axial direction is 16000 nm, almost 24 times of the ideal axial sampling size, an undersampling problem was caused. As a consequence, the performance of the deconvolution was degraded as it lacked necessary information to compute the correct PSF, and the segmentation and reconstruction results were not optimal. In this thesis, we could not intensively evaluate the influence of undersampling on deconvolution, segmentation and reconstruction due to the limitation of the microscope system. Nevertheless, it should be taken into account in future research.

Oversampling is the effect of having an actual sampling size that is smaller than the ideal sampling size. Excessive oversampling in z-direction would significantly increase the imaging time and number of image slices that need to be processed. However, the impact of oversampling on the image analysis results is still unknown. To assess this impact, in **chapter 4** we compared the phenotypic parameters measured from the wide-field microscope image stacks acquired using different z-sampling sizes (5000nm, 10000nm, 15000nm and 20000nm) with the same parameters measured from the confocal microscope images with z-sampling size of 5000nm. To our surprise, oversampling by using z-sampling size 5000nm did not help us to get more comparable results to the confocal microscope. Compared with the z-sampling size 15000nm, the

differences of parameters between the wide-field microscope images with z-sampling size 5000nm and confocal microscope images are bigger, indicating that oversampling does not only increase the imaging time, computational complexity and image storage capacity, but also impairs the quantification results.

6.2.3 Image analysis modalities: 2D projection or 3D reconstruction

To investigate the phenotypic organization of 3D cultured micro-tissues for high-throughput screening, we developed two image analysis platforms to perform high-content analysis. The platform described in **chapter 3** started with a 2D projection process to collapse each image stack into a single image slice, and followed by phenotypic profiling based on this single slice. The second platform described in **chapter 5** was able to reconstruct 3D structures from image stacks. Subsequently, phenotypic profiling was performed either on the reconstructed structures or directly on the image stacks. The advantage of the 2D projection platform is firstly reflected in the imaging time and image size. 2D projection does not require ideal z-sampling size and therefore we set up a z-sampling size (50000nm) much bigger than the ideal z-sampling size (16882nm), which significantly reduced the imaging time, as well as the image stack size. Another advantage is computational simplicity. Without computational expensive procedures such as deconvolution, 3D segmentation and 3D reconstruction, the whole analysis process is fast and not limited by available computer memory or processor speed. We tested this platform on a high-content screening of mouse breast cancer cells (4T1 cells) in a 384-well plate format using an Intel i7 2600 model with 16 GB of RAM and Windows 7 64-bits operation system. Our complete method took approximately 280 minutes of computational time for processing 384 wells, this is much faster than the analysis performed with our 3D analysis platform which took 1150 minutes for the same number of wells. The disadvantage of this platform is the fact that it discards the depth information of tissue development, which may significantly influence the phenotype characterization and classification. Our second, 3D analysis platform fills this gap by establishing a real 3D analysis platform that is able to reconstruct 3D micro-tissue structures and measure phenotypic parameters based on 3D information. However, this achievement is at the cost of almost 5 times the computational time that is needed to complete the 2D projection based platform for processing 384 wells. Furthermore, how crucial the information in z-direction that this 3D platform delivers in phenotype characterization and classification of mouse breast cancer 4T1 cells, is unknown. To answer this question, in **chapter 5** we statistically assessed the performance of the two image analysis platforms in a 4T1 cells screen, where the 4T1 cells were cultured in 384-well high-content imaging micro-plates and exposed to 12 compounds at 6 different concentrations in quadruplicate. We firstly compared the sensitivity of both platforms according to their ability to identify active concentrations of compounds (also referred to as "hits") that significantly affect the invasion of the 4T1 cells. As a result, more concentrations were identified as effective using the platform based on 2D projection, reflecting its higher sensitivity in terms

of hits identification. Secondly, we labeled the compounds according to their biological activity and applied various classification techniques based on the phenotypic parameters. The classification accuracy of the 3D platform was slightly higher than the 2D projection platform, indicating a benefit of incorporating spatial information in z-direction for phenotype characterization and classification. In the end, we tested both platforms on the reproducibility of concentration dependent phenotypic trajectory for each compound. The method to model the phenotypic trajectory is described in **chapter 3**. The 3D analysis platform provided much higher reproducibility than the 2D projection platform, further confirming the superiority of the 3D analysis in respect to phenotype characterization.

6.3 Ultra-high content analysis

Advances in high throughput technology have enabled collecting thousands of images from large-scale screens in a single day [7–10]. However, the image analysis technique is still a bottleneck. Several pioneering screens have either relied on visual scoring by experts [11, 12], or developed their in-house automated analysis software to measure single or at most few phenotypic parameters [13]. Those parameters are often too specific for a certain type of phenotype and hence not suitable for other screens. Moreover, those methods largely depend on the expertise of biologists, but often even biologists are not certain of all possible effects. Consequently, many details were lost and subtle changes may not have been detected. In this thesis, our goal was to develop ultra-high content analysis platforms which should be able to collect maximum information of phenotypes from images, and applied for various types of screening assays. Compared to other existing high-content analysis platforms, the novelties of our platforms were presented in its ability to extract subpopulation information and its ultra-high level in information content.

6.3.1 Subpopulations

Many researchers have found that responses of cells are inhomogeneous [14–16]. For example, in **chapter 2** we showed that not all HepG2 cells respond to the TNFα stimulation synchronously. Therefore, subpopulations should be taken into account and this requires single cell measurement. In this thesis, all developed image analysis platforms are able to perform single-object measurements in order to extract inhomogeneous, cell subpopulation information. In **chapter 2**, we firstly measured the time course of NF-κB nuclear translocation for each single HepG2 cell. Afterwards, the cells were categorized into different subgroups according to the number of translocation peaks, and finally cell subpopulations were investigated before and after TNFα stimulation. In **chapter 3**, we embed an automated classification system in the developed platform to automatically distinguish the spherical cell clusters and branched cell networks. Subsequently, the subpopulation related parameters were collected and included as a part of phenotypic profiling. In order to assess the value of these subpopulations information, we performed compounds clustering and classification with

and without subpopulation parameters on the 3D cultured 4T1 cells screen, where cells were exposed to 29 compounds that can be categorized according to their biological target. The result showed that excluding subpopulation parameters resulted in a failure to co-cluster compounds with the same biological activity and decreased classification accuracy. Similarly, the 3D analysis platform developed in **chapter 5** classified cell clusters to either spherical structure or branched structure according to the sphericity and number of branches. Extracting subpopulation related parameters and using them for compounds classification increased the classification accuracy, further confirming the importance of subpopulation information and which should be taken into account for phenotype characterization.

6.3.2 Ultra-high level in information content

Most of NF-κB translocation studies only investigate the dynamics of NF-κB nuclear translocation through the time course of translocation profiles. We have extended our system so that it can automatically quantify 26 analogue parameters for each individual translocation profile, such as the number of translocation peaks and time between consecutive peaks [5]. These parameters can be used to distinguish inhomogeneous cell populations and might be a powerful tool to study heterogeneous cell behavior in the future. Both image analysis platforms we developed in **chapter 3** and **chapter 5** can measure not only classic morphological parameters, but also topological parameters, intensity properties, texture, moments, as well as subpopulation information, therefore providing the full spectrum of phenotypic information. This is not only beneficial for detecting subtle changes that would otherwise be missed, but also enables system-level studies of the full range of phenotype characteristics. For example, we have showed in **chapter 3** that the concentration dependent phenotypic trajectory for each compound tested in the 4T1 cells screen could be modeled based on the full spectrum of phenotypic characteristics. We also used the phenotypic trajectories to successfully co-cluster compounds with the same biological activity. For both the 2D projection based platform and 3D analysis platform, the compounds classification according to their biological activity was achieved with high classification accuracy.

Another advantage of measuring the full spectrum of phenotypic information is robustness. It enables us to analyze 3D-cellular phenotypes under a wide variety of conditions. In **chapter 3**, we tested our analysis platform on a high-content screening of 44 known human breast cancer cell lines that have been categorized as basal-A, basal-B, luminal or luminal/ERBB2+ based on their gene expression profiles. The result showed that those cell lines can be correctly classified based on the phenotypic profiling with a classification error rater less than 6%. Although initially the use of all parameters may seem redundant, our feature selection methodologies automatically identify those features that contribute most to the separation and characterization of the particular phenotypes under study. In addition to the initial application of our analysis platforms in the 4T1 cell screen and classification of 44 human breast cancer cell lines, described in **chapter 3 and 4**, they have been successfully used in various other screens, such as compound screens in invasive prostate cancer cells and

invasive lung cancer cells (not yet published), confirming their robustness and their wider applicability.

6.4 Future perspective

With the successful development of advanced image analysis platforms for high-throughput and high-content screens, we are able to extract a wealth of information from screening images. The platform developed for analyzing NF-κB nuclear translocation in **chapter 2** can provide a time course profile of translocation dynamics and 26 analogue parameters for each individual cell, as well as for the whole population. In **chapter 5**, the 2D projection based platform we developed to profile the phenotype of 3D cultured micro-tissues can measure up to 794 parameters, while the 3D analysis platform can measure up to 290 parameters. Now the bottleneck is moving downstream to the data analysis and data mining. How to fully explore these rich datasets to reveal cellular signaling networks, is a challenge. Most of the data analysis methods for high-throughput and high-content screening still remain at the stage of hit identification. Quality control such as evaluating *Z'-factor* based on a single parameter is already out of date. For data mining, we showed a few applications in this thesis. For example, we applied phenotype clustering and classification on the 3D cultured 4T1 cell screen to characterize the compounds according to their biological target. For the NF-κB nuclear translocation assay, we applied this method to identify novel regulators of TNFα-induced apoptosis in human HepG2 cells [17]. However, more powerful tools and systematical methodologies are still needed to relate phenotype characteristics to cellular pathways.

In order to extract reliable information from microscope images in an efficient way, more efforts are required to improve current image analysis methods for high-throughput and high-content screening. For example, the platform developed in **chapter 3** still requires human interaction to train an embedded classifier for recognizing different subpopulations, though human interaction is needed only once for the screening of all similar phenotype structures. A built-in clustering which is able to automatically group subpopulations would improve the automation level so that human labor is not required in the whole image analysis process. Moreover, potential subpopulations which are not identified by human vision would be detected. In **chapter 4**, a simple method to correct nuclear size in z-direction was performed using images of fluorescent beads with known size. However, more advanced methods to normalize the dimension of multi-cellular structures despite of their irregular size and shapes are still needed. Another direction to improve our image analysis methods is to increase the analysis time efficiency, where enabling multi-thread processing on a computer cluster would greatly contribute.

Although the impact of imaging resolution on the quantification result was not extensively investigated in this thesis, we should bear in mind that different imaging modalities and image resolutions would generate different quantification results and therefore may lead to a different conclusion. In **chapter 4**, we showed that different z-

sampling sizes influenced the morphological parameters, topological parameters and moments obtained from our image analysis platform, and therefore an optimal z-sampling size was defined for high-content screening of 3D cultured micro-tissues. In the future, sampling in axial direction should also be optimized, as well as other imaging setting.

The integration of high-throughput and high-content screening with other omics technologies is getting more and more attention. For example, a study recently integrated a high-content RNAi screen with phosphoproteomics and transcriptomics to unravel DNA damage response signaling processes in mouse embryonic stem cells treated with cisplatin [18]. Another example is the integration of our NF-κB nuclear translocation assay in a RNAi screen to identify novel regulators of TNFα-induced apoptosis in human HepG2 cells [18]. This study used an in vitro liver cell model and methods such as high-content imaging and functional genomics, which brought us closer to understanding the molecular mechanisms of chronic inflammation and drug-induced liver injury. All those studies showed that combining different omics data with high-throughput and high-content screening would piece together a more comprehensive picture of signaling networks. Furthermore, fueled with mathematical modeling, integration of high-throughput and high-content screening with other omics technologies would open a new era for systems biology.

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