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A high-content screening technology for quantitatively studying podocyte dynamics

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Abstract

Podocytes form the visceral layer of a renal glomerulus and express a characteristic octopus—like cellular architecture specialized for the ultrafiltration of blood. The cytoskeletal dynamics and structural elasticity of podocytes rely on the self-organization of highly interconnected actin bundles and the maintenance of these features is important for the intact glomerular filtration. Development of more differentiated podocytes in culture has dramatically increased our understanding of the molecular mechanisms regulating podocyte actin dynamics. Podocytes are damaged in a variety of kidney diseases and therapies targeting podocytes are being investigated with increasing efforts. Association between podocyte damage and disease severity—or between podocyte recovery and the performance of therapeutic molecules—have been the venues of research for years. In this perspective, more standardized high content screening (HCS) has emerged as a powerful tool for visualization and analysis of podocyte morphology. This high-throughput fluorescence microscopy technique is based on an automated image analysis with simultaneous detection of various phenotypes (multiplexing) across multiple phenotypic parameters (multiparametric). Here, we review the principles of HCS technology and summarize efforts to carry out small compound screen using podocytes.

Introduction

Urine formation starts with the filtration of blood through the glomerular filtration barrier, which is a three-layer interface formed by fenestrated endothelial cells[1], glomerular basement membrane (GBM)[2], and visceral epithelial cells, also known as podocytes[3]. Owing to its delicate structure and coordinated cell polarity, glomerular filtration barrier constitutes a size and charge selectivity that facilitates cation transport and filters all the small molecules (water, salt, glucose, amino acids, urea, etc.) out of the blood but retains cells, platelets and large proteins, such as albumin.

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As a key player of the glomerular filtration, podocytes are aligned on the external surface of GBM and cover glomerular capillaries neatly with numerous cytoplasmic projections, called foot processes (FPs)[4]. These terminally differentiated epithelial cells form the final barrier to protein loss by interdigitating with the FPs of the adjacent podocytes and leaving between them the slit diaphragm (SD), narrow filtration slits that are bridged by modified adherens junction[5]. Podocyte's functions, which are vital to glomerular filtration, depend on a highly regulated actin cytoskeletal network that is formed either by a central actin bundle along the long axis of FPs or by a relatively short cortical network aligning at the cell periphery and anchoring the components of SD[6].

Podocytes are the major targets of several agents or molecules such as toxins, reactive oxygen species (ROS), complements, and antibodies[7]. Injury to podocytes may physically alter this elaborate structure causing the flattening and retraction of the FPs as well as the disappearance of the filtration slits, a process called FP effacement. Therefore, efforts to reverse the podocyte damage and rescue glomerular filtration generally focus on actin regulatory pathways[8] and developing therapeutic agents that can ameliorate disruptions of actin organization[9].

A successful disease-specific tailoring of therapeutics may be achieved by using an image-based screening, which enables to analyze a wide variety of phenotypes in cells. Such high content screening (HCS) platforms employ fully automated microscopes and image analysis software, making it possible to quantify changes in cellular and subcellular properties including cell area, morphology, actin fiber and focal adhesion intensity. We recently described a novel phenotype-based HCS using immortalized mouse podocyte cells and applied it to identify podocyte-protective small molecules[10]. This review aims to discuss the screening experiments and image analysis approaches, as this high-throughput technique is being used in the preclinical development of the drug discovery process.

PODOCYTE AS A DIRECT TARGET OF DRUGS

Kidneys have arguably the most complex membrane system and solute trafficking in the body, which attracted researchers with interests in kidney biology for many decades[11]. This is mostly due to the multicomponent nature of the glomerular filtration system, with endothelial cells, glomerular basement membrane (GBM), and visceral epithelial cells (podocytes) participating in the filtration process[4]. The function of this elegant filtration system is maintained by the interplay among these core constituents as well as the immaculate arrangement of the structural proteins within the membrane. The integrity[12] and elasticity[13] are other fundamental concepts since the capillary pressures far exceed those in other organs. The mechanical support required for glomerular capillaries are mainly provided by podocytes[14] since GBM and its associated cells are not rigid, but rather flexible[15]. Furthermore, endothelial cells lack sufficient cytoskeletal structure (and contractile system) as demonstrated by the electron microscopy[16]. Hence, among the principal components of the glomerular filtration barrier, podocyte deserves a special attention. And it really has: during the past decade, podocyte research has remarkably expanded, with more than 3000 published papers directed toward delineating the mechanisms regulating podocyte structure and function.

Owing to its strategic location, podocyte is the major target of various agents soluble in the blood, including toxic and immunologic compounds, reactive oxygen species (ROS), complements, and antibodies to podocyte membrane antigens[7]. Podocyte is also injured by other means, such as genetic deletions or mutations impacting the proteins of podocyte itself, SD complex and GBM structure or charge distortion directly affecting its apical membrane domain[7]. Podocyte injury leads to reorganization of actin cytoskeleton from a dynamic state (characterized by parallel and contractile actin filaments) to a rigid state (represented by thicker stress fibers) and foot process (FP) effacement (fusion or retraction of podocyte terminal processes)[17]. Beyond these structural changes and phenotypic conversions, persistent injuries to podocyte might cause lethal alterations such as detachment from the underlying GBM[18] (as a relevant note, podocytes disappearing from the glomerular tuft can be still alive and recovered from the urine[19]) and death[20]. The loss of podocytes is an irreversible event causing the loss of glomerular filtration function since podocytes are post-mitotic cells with a minimal capacity to replicate[21]. Once podocytes are lost, the remaining podocytes fail to completely cover the outer aspect of the GBM and become more vulnerable to any additional workload[22]. Potential mechanisms for podocyte replacement include the contribution of glomerular parietal epithelial cells (PECs) and cells of renin lineage (CoRL) as podocyte progenitors; however, uncertainties still remain regarding the routes of the migratory event that brought those progenitors to the glomerular tuft and the formation of complex cytoskeletal structure (i.e., FPs and SDs) in these candidate cells[23].

Notably, there is abundant evidence indicating that any abnormality or change in podocyte cytoskeleton may contribute to proteinuria and nephrotic syndrome[24], and more strikingly, disorders affecting glomerular filtration are responsible for 90% end-stage kidney diseases (ESKD) at a cost of $30 billion per year in the US alone[25]. Several pharmacological agents targeting podocytes are being evaluated such as corticosteroids, angiotensin I-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), peroxisome proliferator-activated receptors (PPAR) agonists, retinoids, and vitamin derivatives[26–28]. Despite tremendous research effort, there are still very limited therapeutic options to stop progressive decline in glomerular filtration rate[9]. Podocytes are therefore a promising target for investigating the pathogenetic mechanisms of renal protection and screening new treatment options.

CULTURED PODOCYTES ARE SUITABLE SOURCES FOR IMAGE ANALYSIS

Fundamental observations of biological processes can be consistently simplified in two dimensional cell culture conditions, where cells grow (and differentiate) in the presence of a defined medium and behave similar to the *in vivo* situation. Regardless of its limitation regarding the representation of the physiological microenvironment, *in vitro* assessment of cell viability, metabolism, and functionality is an important step to many aspects of biomedical research. Therefore, over the years, traditional *in vitro* cell culture helps researchers to predict the response of more complex organisms (e.g., tissues, organs) to potential pharmaceuticals.

Kidney diseases are mainly characterized by structural and functional changes in glomerulus that causes a gradual loss of kidney function (measured by glomerular filtration rate, GFR) and terminal renal failure, if not treated promptly. Physical properties of cells constituting glomerular filtration barrier have been shown to have a significant role in various kidney diseases, as in many instances podocyte cytoskeleton is disorganized and podocyte adhesion decreased when the cells are no longer healthy. In that sense, the availability of murine[29] and human podocyte[30] cell lines is crucial since the injury-induced cytoskeletal changes and injury-driven cell motility can be evaluated by well-defined protocols in cultured podocytes.

Using a temperature-sensitive transgene, conditionally immortalized podocytes are able to proliferate under permissive conditions (at 33°C and with the presence of interferon-γ □□ podocytes are of mouse origin) and display characteristic cobblestone shape, whereas under non-permissive temperature (37°C), the cells stop replicating and starts differentiating by developing a large arborized morphology containing well-developed processes[31]. Actually, this one-cell-thick podocyte monolayer cannot fully replicate the highly sophisticated *in vivo* kidney filtration barrier (i.e., neither podocyte-GBM interaction is represented with a great molecular detail by the interaction of podocyte and extracellular matrix (ECM) ligands in the tissue culture flask, nor the tight connections of podocytes with each other through specialized cell-adhesion molecules *in vitro* is really an accurate cellular representation of filtration slits) but still offers a quite successful research venue for mechanistic studies. This is mainly due to their ability to express virtually all podocyte-specific markers[31] such as synaptopodin, which is an actin-binding protein and involved in cytoskeletal organization[32]. Synaptopodin is profoundly expressed in podocyte FPs *in vivo* and synaptopodin-deficient mice abrogates stress-fiber formation and has an impaired recovery from proteinuria[33]. Since most glomerular diseases are characterized by FP effacement leading to proteinuria[8], loss of stress fibers or reduced expression of synaptopodin in cultured podocytes can serve as surrogate markers for these pathological events *in vitro*. Rearrangement of actin cytoskeleton also modulates cell-matrix adhesion and plays a direct causative role in development of migratory phenotype, which can be analyzed *in vitro* by a functional podocyte assay[34]. If performed on a large scale by HCS platforms, these well-designed assays and related phenotypic measurements prepare the stage for potential clinical translation.

HIGH THROUGHPUT PLATFORM FOR STUDYING PODOCYTES

Over the last 20 years following the establishment of immortalized mouse podocyte cell line, it became more obvious that podocytes—as targets of various pathogenic pathways—have a key function in kidney diseases, regardless of the initial etiology, and cultured podocytes have potential to provide a suitable informational content to explore the molecular mechanisms mediating podocyte function and to validate kidney disease models. However, our capability to evaluate effective therapies to kidney dysfunction has somehow been limited by the poor translation of phenotypic changes to disease outcomes. In preclinical studies, it is particularly important to track changes in phenotype over time to better characterize the cellular responses to various external factors. To this end, high-throughput platforms using animal- or human-derived cell models are integral to facilitating the efforts to investigate the function of cells in disease and to improve our ability to predict whether

the compounds of interest would lead to clinically useful drugs, irrespective of the level of disease complexity.

High content screening (HCS) combines the power of automated fluorescence microscopy and image analysis software for quantitative and dynamic measurements of the biological changes in cells[35,36]. The algorithm includes assay design, image acquisition, image analysis, and data interpretation. The major advantage of this technology is its ability of multiplex profiling (i.e., detection of diverse cellular states simultaneously) and multiparametric analysis (i.e., measurement of multiple phenotypic parameters at the same time) in an automated and robust manner. Applied to drug discovery, HCS campaign allows us to discover new compounds that are capable of enhancing cell's stress response, improving the phenotype, and preventing an additional damage[37,38].

We recently developed a podocyte-based HCS assay[10] to screen compounds for their antiproteinuric activity (see Figure 1 for the schematic overview of experimental workflow). We took advantage of using immortalized murine podocytes[29], which are highly proliferative when cultured under permissive conditions and exhibit the characteristics of differentiated podocytes with elongated cellular processes protruding from the cell body and concomitant expression of synaptopodin and other podocyte marker proteins such as nephrin, podocin, CD2AP, TRPC6, α3βi integrin, and α-actinin-4[31]. The initial effort started with standardizing the cell culture conditions (passage number, seeding density, plate type, media, ECM composition, etc.) to eliminate assay-to-assay variability and make historical performance comparisons easy.

We used puromycin aminonucleoside (PAN), a well-established rat model of proteinuria[39] and a commonly used experimental model of podocyte injury *in vitro*[34] to induce podocyte damage, which is manifested by the appearance of a relatively high proportion of rounded cells with reduced actin fibers and focal contacts and weak adhesion to ECM[40,41]. Following the treatment of cells with varying doses of PAN, we captured representative images of hundreds of cells simultaneously in an automated fashion. Then, we quantified the cellular phenotypes (i.e., cell size and morphology, intensity and spatial distribution of F-actin fibers and focal adhesions) in an unbiased manner by the computer-assisted image analysis (Figure 2), which is the key step in the protocol since it translates the assay readout into podocyte response and function using the immense amount of information stored in these microscope-generated images[42]. PAN-mediated podocyte injury can be ameliorated by several pharmacological agents, including dexamethasone, fluvastatin, darbepoetin, mizoribine, sialic acid, and nuclear factor kappa B (NF-κB) inhibitor dehydroxymethylepoxyquinomicin (DHMEQ)[7]. Among those, we chose mizoribine (MZR)[43] and demonstrated that we were able to protect podocytes and rule out the adverse effects of PAN on podocyte cytoskeleton by MZR treatment. To validate our findings with a relevant protective agent, we used the glucocorticoid dexamethasone (DEX)[44] and treated podocytes with varying doses of DEX to investigate its efficacy against PAN. We consistently obtained similar results toward the recovery from PAN-induced injury from each of the agents we employed, highlighted with the increased F-actin fibers and focal adhesions, higher synaptopodin levels and decreased cell roundness. This suggests that our HCS methodology is reliable across plates and experiments and not limited to a single model.

We further verified the assay performance by testing the reproducibility and robustness of our HCS algorithm. In particular, we determined Z′ factor across multiple replicates on an analysis plate[45]. It is a statistical test used to compare the conditions (e.g., positive and negative controls) and indicates a better separation (with a low standard deviation) between these groups when it approaches 1.0[45]. It is suited to most HCS readouts to assess the quality of screening assays[46]. Repeating the multiparametric analysis for untreated and PAN-treated podocytes resulted in Z′ values of ≥0.46 and 0.44 for changes in cell roundness and F-actin fibers, respectively, indicating the robustness and reproducibility of our assay.

Based on the aggregated data (from individual cells to treatment wells) and the dynamics of the injury and recovery process, we chose cell roundness as the major feature to use at our primary HCS assay employing the library of 2121 pharmacologically active compounds. Approximation to one-dimensional parametric analysis improved the efficiency of our HCS campaign by speeding the screening and reducing the data storage. This single parameter approach is also applied to perform phenotypic screening to detect drug candidates[47] or RNAi libraries[48] in cancer pharmacology, where cell viability represents the reliable readout. Here, the ultimate goal was to screen those compounds on PAN-treated podocytes and identify hits (compounds exhibiting the protective properties) for additional testing. Our analysis resulted in a primary hit list of 24 compounds (with approximately 1% hit rate), which were re-analyzed using independently obtained powder forms. Our strategy here was to cluster the top primary hits in functional groups and start with the ones having potential protective roles for significant podocyte-related proteins. Among those, the small molecule β1-agonist pyrintegrin drew our attention due to a recent study reporting that pyrintegrin increased cell-ECM adhesion-mediated integrin activity in human embryonic stem cells (hESCs), in which β1 integrin is regarded as a major integrin[49]. Almost a decade ago, we had shown that PAN-treated mouse podocytes demonstrated a marked down-regulation of α3β1 integrin[34], which is the anchoring unit of podocyte FPs to GBM[50]. Podocyte-specific deletion of β1 in mice leads to severe proteinuria as early as 3 weeks of age[51] and death within 15 weeks[52] indicating the critical role of α3β1 integrin in maintaining the structural integrity of the glomerulus. In the light of these findings, we were able to identify β1-agonist pyrintegrin as a podocyte-protective agent by HCS and validated it *in vivo* (in the setting of PAN-induced nephropathy in rats).

Overall, our HCS platform had the unique ability to quantify multiple phenotypic properties of podocytes with high sensitivity and identify the repertoire of small molecules preventing PAN-mediated podocyte disease. This fully automated and computer-aided analysis platform will accelerate the progress exploring therapies for preserving glomerular filtration function.

Conclusion

Podocytes are highly differentiated epithelial cells covering glomerular capillaries of the kidneys. They are well decorated with a dense array of stress fibers containing F-actin and hence have elasticity and contractile properties to bear the excess capillary pressure exerted by blood (filtrate) on the glomerular capsule. Several molecular pathways directly or indirectly contribute to podocyte injury, which is a common denominator of various glomerular diseases associated with proteinuria. Unfortunately, unlike other self-renewing

epithelial cells, podocytes have a very limited ability to regenerate. Therefore, once podocyte cytoskeleton is altered or podocyte loss starts, these pathologic states operating in conjunction with each other further aggravate podocyte injury. This is clinically significant because of their critical role in keeping the glomerular filtration functioning properly. Knowing that podocytes are sensitive to many pathological conditions that lead to kidney damage, we aimed to develop a high-throughput phenotypic assay to study the ameliorative effect of a library of compounds on cultured podocytes in the context of a well-characterized glomerular disease model. We presented a fully automatic and quantitative image analysis approach that exploits multiple morphological details simultaneously at a single cell level. To our knowledge, this is the first HCS algorithm applied to podocytes and it is applicable to other physiologically relevant systems (i.e., systems designed to promote podocyte repair from other injury-inducing factors or to protect other glomerular cells from similar proteinuric agents) after an appropriate modification and agent-or cell-specific testing. Indeed, in pursuit of this methodology, HCS technology was used to screen compounds that reduced podocyte migration for the purpose of identification of potential therapeutic targets in a recent study[53,54]. These multi-parametric screening approaches, together with supporting studies, will enhance our systematic understanding of the regulation of podocyte cytoskeleton, function, and survival as well as basic mechanisms of glomerular diseases and drug actions.

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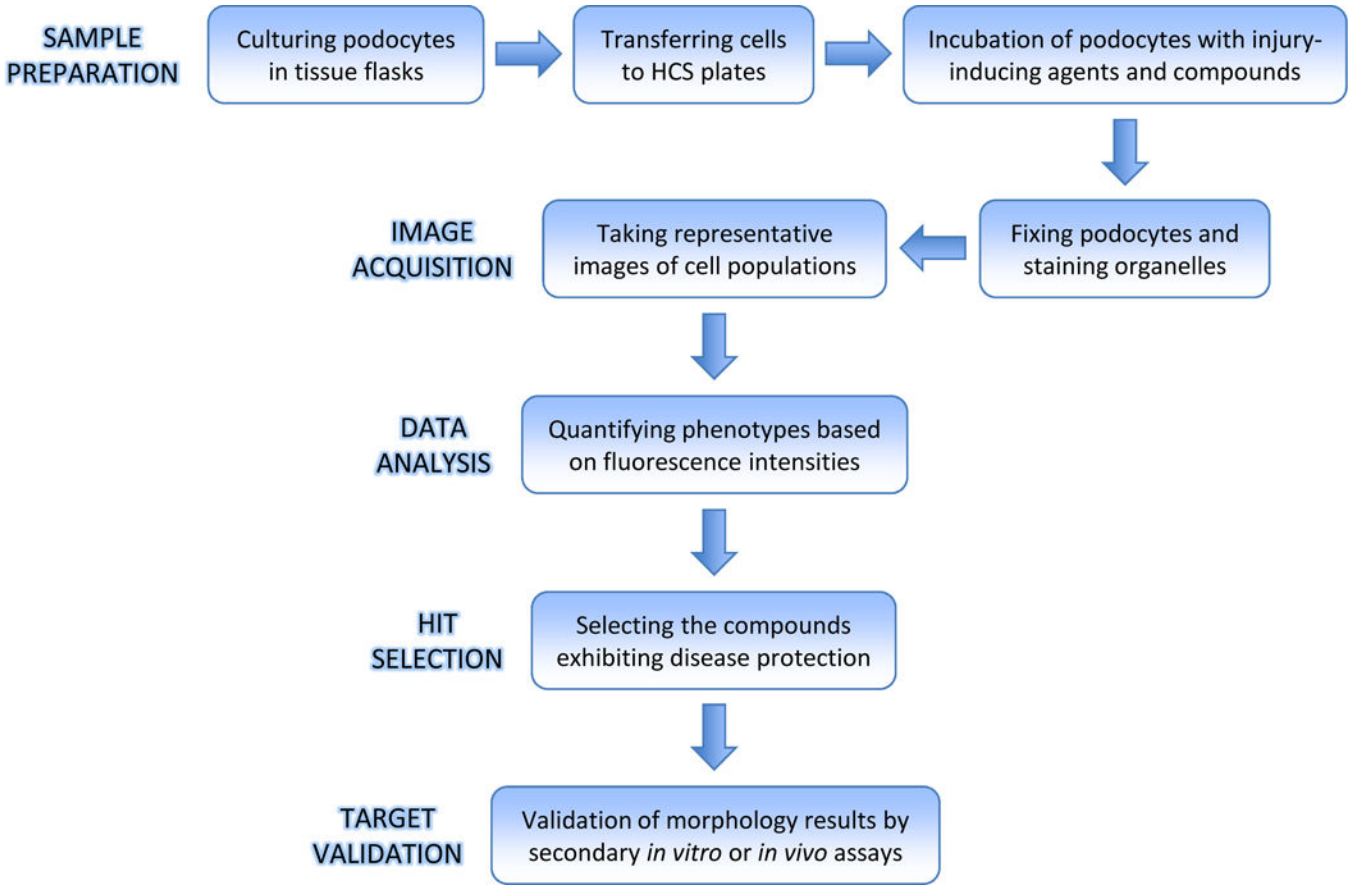

Figure 1. Set up of the podocyte-based HCS platform

Immortalized mouse podocytes are proliferated at permissive temperature (33°C). When the culture reach 75 to 80% confluency, podocytes are harvested and cultured in tissue flasks at 37°C for differentiation. After 7 days, cells are detached and transferred to multiwell plates in which they are further cultured at 37°C for 4 days. Podocytes are treated with disease agent (PAN) and the library of compounds and fixed at the end of the treatment period. The nuclei, cytoplasmic boundaries, actin fibers, and focal adhesions are stained before acquiring images from each well of the plate on fully automated HCS system. Image analysis is performed using high content analysis software to examine (i.e., quantify) cell phenotypes. New targets (hits) are identified by screening compounds with desired mechanisms of action, i.e., alleviating adverse phenotypes. The biological activities of the most promising targets need to be further validated by secondary *in vitro* and *in vivo* assays before being introduced into the clinic.

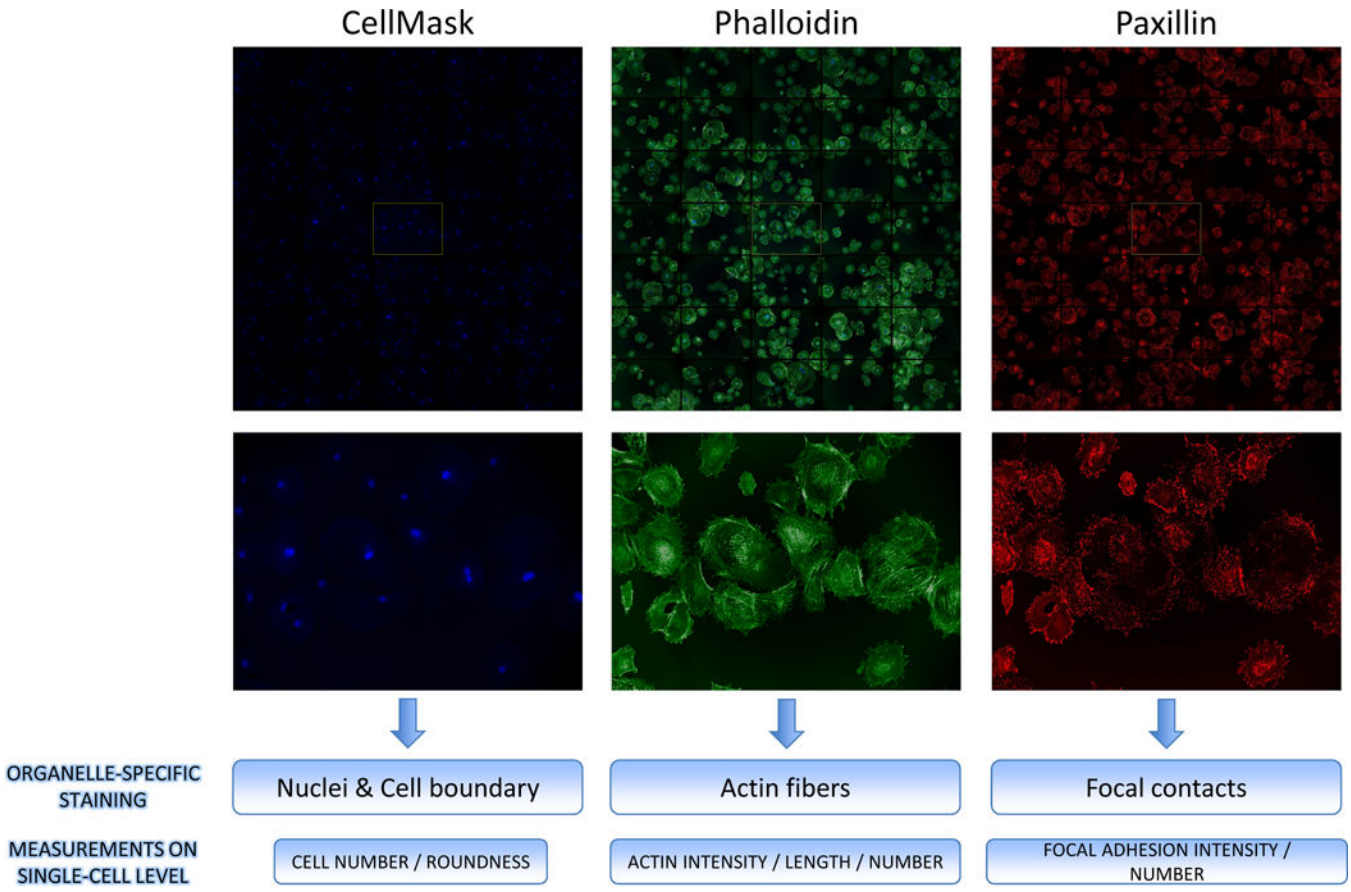

Figure 2. Image analysis of a podocyte phenotypic assay

Prior to imaging, nuclei and cytoplasmic boundaries are stained with CellMask (blue), F-actin is stained with phalloidin (green), and focal adhesions are stained with paxillin antibody (red). Cellular phenotypes are calculated on a single-cell level by quantitative imaging platform of HCS system. Additional details regarding the staining and image analysis protocols can be found in Lee *et al.*[10].