Label-free characterization of organoids with quantitative confocal Raman spectral imaging

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SUMMARY: Stem cell derived organoids have the potential to significantly improve the drug discovery process. However, a key challenge is monitoring the maturation process and drug response. LaLone et al. show that quantitative confocal Raman spectral imaging, a label-free technique, can reliably monitor organoid development, drug accumulation, and drug metabolism.

MAIN TEXT: Extrapolating results from classical cell line and animal model systems to humans has become a major bottleneck in the drug discovery process. Two-dimensional cell cultures are not representative of real cell environments, while several studies have identified biological processes that are specific to the human body and cannot be modelled in animals [1]. Organoids, which are miniature self-organized three-dimensional tissue cultures derived from stem cells, have the potential to overcome these limitations. Organoids can be crafted to replicate much of the complexity of an organ, and their potential to extend basic biological research, medical research, and drug discovery into a more physiologically relevant human setting is becoming widely appreciated. One feature that is common to all organoids is that they are generated from either pluripotent stem cells (PSCs) or adult stem cells through replicating human development or organ regeneration in vitro [2]. However, the creation of human adult stem cell-derived organoids is limited by accessibility to the tissue and prior knowledge of the culture conditions for that tissue, while an iPSC line, once derived from a patient, can be used to repeatedly generate different tissue models without any time limit [3].

Numerous PSC-derived organoid models have been developed, including intestinal organoids [4], optic cup organoids [5], and brain organoids [6]. A key

challenge with developing any organoid model is monitoring the maturation process or the response of organoids to changes in environment. In general, several markers at the gene, protein, and functional level must be screened simultaneously. However, most of the methods that are employed, including fluorescent imaging, lack the ability to measure several markers at once. As a result, large sample volumes are required, which can be problematic given the heterogeneity of organoids. An alternative approach is to use a label-free method [7, 8], which allows numerous markers to be interrogated simultaneously. One such method is Raman spectroscopy, which is sensitive to the vibrational modes of molecules, and as a result, can enable label-free visualization of a wide range of molecules (including carbohydrates, lipids, proteins, nucleic acids, specific metabolites, drugs, and minerals) in biological specimens.

Research led by Molly Stevens and Stefan Krauss, published in *Cell Reports Methods*, has shown that quantitative confocal Raman spectral imaging, or qRamanomics as denoted by the authors, can provide quantitative spatial chemotyping of major classes of biomolecules in liver organoids [7]. The experimental setup, which integrates confocal microscopy and Raman spectroscopy, allows Raman scattering from within a confocal volume to be detected (Fig. 1). A spectral unmixing algorithm then enables direct classification of a broad range of biomolecules contained within the volume, including different classes of lipids, cytochrome C, proteins, nucleic acids, glycogen, vitamins, and selected xenobiotics. Scanning the beam through the sample provides a three-dimensional map of the individual biomolecules, allowing both the concentration and location to be easily quantified. The technique was initially validated by measuring primary human hepatocyte spheroids (3D PHH), and comparing the results with labelled confocal images obtained from similar 3D PHH showing albumin, neutral lipids, and DNA. Following this, Raman data from iPSC-derived hepatocyte-like organoids (3D iHLC) and 3D PHH were obtained to investigate the potential of the technique to serve as a benchmarking tool for 3D liver representations. Statistical differences between 3D PHH and 3D iHLC were seen in protein amounts, nucleic acids, and unsaturated lipids, suggesting that these markers could be useful benchmarking tools. The true benefit of the scanning confocal experimental setup was then demonstrated by

producing heat maps representing correlations between component spectra in each confocal volume. This allowed tendencies for spatial co-localization of biomolecules to be quantified. Interestingly, saturated lipids were to a higher degree colocalized with proteins in 3D iHLC compared to 3D PHH. In contrast, nucleic acids more frequently colocalized with proteins in 3D PHH compared to 3D iHLC.

While employing quantitative confocal Raman spectral imaging as a benchmarking tool is certainly an important application, the vast potential of the technique was demonstrated by the measurements carried out on organoids following drug exposure. 3D PHH and 3D iHLC were exposed for 48 hours to 10 μM amiodarone, nilotinib, fluticasone-propionate, ketoconazole, or methadone, and subsequently analyzed by the developed technique and conventional assays. Notable differences were observed between the treated and untreated groups, showing that label-free Raman imaging can aid the drug discovery process. Most striking though was the measurements of drug and metabolite accumulation in organoids. Here, the unique Raman fingerprints of the individual drugs allowed a map of their accumulation to be created. Spatial colocalization within protein-rich voxels indicated metabolic processing, an important insight to gain. Comparison of unmixed deposit spectra with parent drug reference spectra revealed differences attributable to changes in molecular bond/structure of the molecule. These spectroscopic changes could be evidence of drug metabolism, which could be a significant insight in the drug discovery process. In general, these experiments show that quantitative confocal Raman spectral imaging provides a label-free spatial and temporal snapshot of the presence and processing of drugs in organoids.

Stevens, Krauss and co-workers also highlight important areas of future development. Standardization will be critical for global scaling of the technique, and this will be needed across a variety of areas. While experimental setups and calibration methodologies will need attention, most important will be standardization of spectral unmixing approaches. Open and transparent algorithms, along with standardized libraries of component spectra will need to be developed to facilitate inter-laboratory comparisons. Additionally, given the well-known heterogeneity of organoids, moving to in vivo measurements would be a powerful

advance, especially when considering the measurements on drug and metabolite accumulations. Overall, the presented technique is a significant addition to the other label-free microscopy-based techniques available for organoid evaluation. Such techniques include coherent confocal light absorption and scattering spectroscopic (C-CLASS) microscopy, which evaluates chromatin state in live organoids [8], and light microscopy-based assays for predicting drug response of organoids [9]. These label-free techniques are an important alternative to fluorescent imaging, given that specificity varies, labeling is time consuming, specialized reagents are required, labeling protocols can kill cells, and even live cell protocols can be phototoxic [10]. Complementary label-free techniques have the potential to significantly advance the monitoring of organoids, leading to better protocols and improved outcomes.

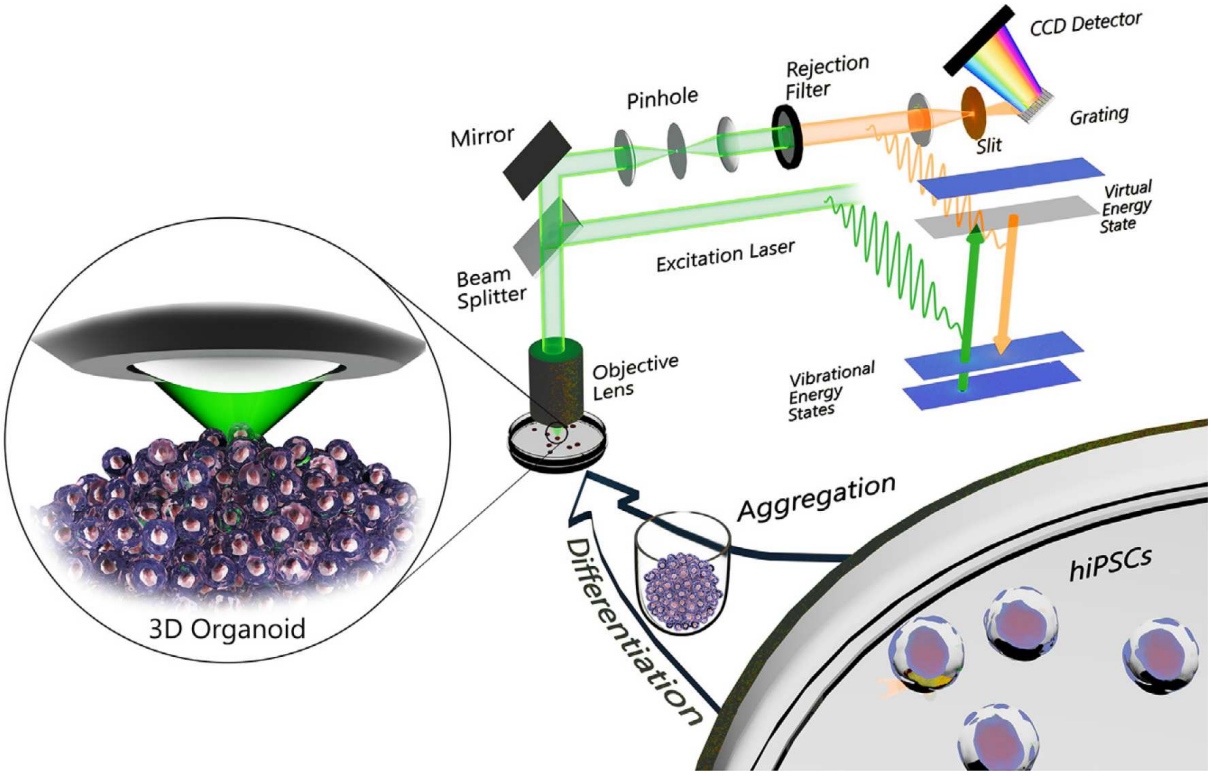

Fig. 1. Quantitative confocal Raman spectral imaging for label-free characterization of three-dimensional organoids. Workflow for the formation of 3D human iPSC organoids (bottom right), along with the schematic of confocal Raman spectroscopic microscope and the basic principles of Raman spectroscopy (top right). The excitation beam of the confocal Raman spectroscopic microscope is delivered to the organoid with a microscope objective and Raman scattering is collected from within the confocal volume within the organoid (inset). Figure created by Umar Khan and Paul Upputuri.

DECLARATIONS OF INTERESTS

The authors declare no competing interests.

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