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Short Commentary

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Image Quantification in Biology: A Short Commentary on Statistical Parametrization of Cell Cytoskeleton (SPOCC)

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Abstract

Imaging and microscopy have played a very important role in biological research. In this commentary, we have provided a summary of the development of different imaging modalities and quantitative techniques as an introduction. We have briefly described the technique Statistical Parametrization of Cell Cytoskeleton (SPOCC) and evaluated it against similar techniques available. We have also discussed the advantages, short comings and future prospective of SPOCC both technically and biologically.

Keywords: *Imaging; Microscopy; Quantification; Correlation; SPOCC*

Introduction

Imaging, as a tool in biological research, has been utilized for a very long time. Immunostaining of biological/pathological samples provide important information regarding the protein expression and distribution [1-3]. The technique evolved from staining with simple dyes to sophisticated fluorophores and fluorescent microscopy, which enhanced the diversity of staining targets, their specificity and the resolution [4]. With the advent of fluorescent proteins, the arena of imaging live biological samples became available [3,5,6]. The next leap forward in biological imaging came with the development of super-resolution techniques such as STORM [7], STED [8], SOFI [9], PALM [10] etc., which improved the image resolution beyond the diffraction limit enabling the study of sub-cellular distribution and patterning of proteins. Using traditional microscopy, it was known that contractile actin stress fibers have both myosin and α-actinin [11-13], but super-resolution uncovered that they form an alternating pattern on the actin fibers [14].

Parallelly with biological imaging, efforts have been made to quantify different aspects of the images which advances the subjective analyses of results into objective measurements. Counting cells [15] stained with a certain dye can uncover how ubiquitous a certain protein is, whereas quantifying the intensity of a certain fluorescently tagged protein in cells can inform regarding the expression level of said protein [16]. In live imaging, cells are often tracked over a period of time in migration and wound healing assays, from which the motility of the cells can be estimated [1]. With higher resolution, sub-cellular processes, such as the expression or reorganization of certain proteins can also be studied and quantified [17,18]. More sophisticated quantitative techniques such as Fluorescence Recovery After Photobleaching (FRAP) [19], Forster Resonance Energy Transfer (FRET) [20], Fluorescence Correlation Spectroscopy (FCS) [21], can advance imaging to quantifying molecular diffusion, exchange and dynamic proximity of a pair of molecules.

The cytoskeleton of a cell has three components: (1) actin [11-13,22,23], (2) microtubules [24,25] and (3) intermediate filaments [26-28]. Their formation and dynamics play a major role in cellular functioning [22]. For example, the actin cytoskeleton is the major contractile and mechanosensitive machinery in all cells [11-13,22], whereas tubulins are implicated in intra-cellular cargo transport with the help of Kinesins [29]. The role of intermediate filaments, though important, can be diverse depending on their type ranging from supporting the actin architecture [30] to formation of agrosomes [31]. These structures are highly dynamic and responsive to external and internal stimuli and a small change in their organization can result in drastic changes in the characteristics of the cell [32], indicating the immense necessity in

better understanding their dynamics, which can be achieved via quantification.

Discussion

In their paper, Basu A, et. al. (2022) developed a novel comprehensive method for quantifying the actin cytoskeleton called Statistical Parametrization of Cell Cytoskeleton [17]. Their work demonstrated the immense importance of such quantification methods as they showed that the organization of the stress fibers affect the mechanical property of cells and are highly correlated with different stages of cancer metastasis (specifically Epithelial Mesenchymal Transition, EMT). In other works, Paul M, et. al. (2019) reported the correlation between premalignant properties of a subpopulation in Human Bronchial Epithelial Cells (HBEC) [33]. In these studies, researchers also correlated the actin organization with genetic pathways responsible in cancer. The method is non-destructive and non-intrusive enabling live imaging of valuable samples and repeated measurements. The other advantage is that the only input required for SPOCC is a simple fluorescent image of the actin cytoskeleton with any type of fluorescent probe, which means that this can be coupled with other imaging-based techniques ranging from motility assays to Fluoresce In Situ Hybridization (FISH) [34] to visualize RNA in the cell.

1. Image Analysis Pipeline

SPOCC is primarily compartmentalized into two parts: (1) Extracting actin fibers from fluorescent image as binarized straight lines with quantifiable length, width, orientation and localization; (2) Evaluating the relative organization of the filaments by calculating Orientational Order Parameter (OOP) [35].

In filament extraction, the first step is to sequester the fluorescent image intro three components. The first component and the main subject of interest is the actin filaments image, the second component is non-filamentous structures, such as aggregates, which still have the same fluorescence signature as the filaments and the third component is noise, which has a much higher frequency compared to the fluorescence signal. The noise component can easily be separated from the other two by simply using a Fourier transform followed by a filter to eliminate high frequency signals. The complication in separating the first two components arises from their similar fluorescence signature and can only be solved by incorporating their structures in the separation pipeline. The filaments by their nature possess a curvilinear nature which is lacked by aggregates and other background structures. Thus, a filter which takes into account the shape of the signal can successfully differentiate between the two. The MCALab library [36] contains two such transforms, the curvelet transform which is used to extract the filaments image and the undecimated wavelets transform which isolates the non-filamentous structures. Multi-scale line segmentation and line-detection algorithms generate the final binarized image and the filament characteristics. OOP is calculated from the individual filament orientations.

There are other techniques that extract filament characteristics and calculate OOP. The most common approach is to perform a Fourier Transform (FT) on individual pixels of the fluorescent image which evaluates the orientation vectors of every pixel. Inspired by Particle Image Velocimetry (PIV) [37], this method breaks down the entire image into a set of overlapping windows which will result in a skewed/ anisotropic FFT signal if the window contains fibrillar structures. Though OOP calculation is equivalent with SPOCC, a few major distinctions exist. As FFT only provides information regarding the existence and direction of anisotropic (fibrillar) structures, it cannot identify individual filaments and thus is incapable of providing filament length or width. Moreover, stress fibers are often thicker than individual pixels (windows) and as such, even though that area is part of a filament structure, the FFT signature will be isotropic. Another approach that can extract similar information but suffers from similar limitations employs pixel intensity gradient [38]. To evaluate orientation vector of individual pixels. This approach

relies on the fact that there should be a sharp drop in intensity at the edge of the filament and the direction of that drop will be orthogonal to the direction of the filament [38]. But apart from the limitation discussed above, this technique has no way of differentiating between filaments and other fluorescent artefacts, which would also have sharp edges and thus introduce errors in the analysis.

In terms of evaluating relative alignment of fibers, the most intuitive approach is to fit the angular distribution to Gaussian or circular distributions and the corresponding standard deviation value will generate information about how narrow the angular distribution, essentially translating into a figure of merit for relative orientation. But fitting the data to any one distribution presumes the knowledge of some empirical model for the formation and organization of fibers. For example, in real cases, the angular distribution can often be non-Gaussian or multimodal which would result in non-negligible errors if that was fitted to a Gaussian distribution. OOP, on the other hand, does not require foreknowledge of any such empirical model or distribution and is free from such fitting errors.

2. Biological Significance

The authors have demonstrated some exciting applications of SPOCC in their works. They have demonstrated that SPOCC can reliably identify and distinguish between different types to cytoskeletal arrangements to the extent of discovering novel phenotypes and identifying sub-populations. The real impact of this method comes from the ubiquitous nature of cytoskeletal structure and dynamics. The actin cytoskeleton, which is present in some form in almost all types of cells, is built from different

types of stress fibers and they contribute uniquely to the relative alignment of fibers. For example, dorsal arcs are less aligned than ventral stress fibers, so it is possible to predict the type of predominant filament in a cell from its OOP value. Also, being the primary mechano transduction and contractile machinery of the cell, the actin cytoskeleton is likely to exhibit the earliest as well as the most drastic reorganization in response to stimuli. So, actin is not only ubiquitous, but its reorganization also is the most sensitive observable phenomenon whenever the cell is undergoing any biological process. Thus, SPOCC, with its ability to quantify the actin cytoskeleton, has the potential to study and quantify, to a high degree, almost any biological process. This is demonstrated in the ability of SPOCC to serve as a marker for EMT progression and to evaluate drug response.

The actin cytoskeleton of the cell is highly correlated with its mechanical properties, such as elasticity and motility. This phenomenon is also validated with the help of SPOCC, where it can be shown that the elasticity of cells with different OOP values are also different; this quantification provides a perfect platform to study the inter-relation of the actin cytoskeleton and cell elasticity.

Conclusion

SPOCC is clearly a very, if not the most, robust actin quantification method. It has the ability to quantify individual filaments in terms of length, width, orientation and location while eliminating fluorescent artefact structures. It can analyze entire fields of view or single cells one at a time. It is important to note that all the structures extracted by SPOCC are straight lines which means that filaments with substantial curvature in them will be broken up into multiple shorter straight lines. Though this generates some benefit in orientational analysis, it is likely to produce some errors in length-bases analyses. Though an algorithm in SPOCC can stitch closely aligned, continuous filaments together, a more sophisticated way of achieving that is required. Also, though SPOCC has certain benefits over other image analysis techniques, it is also lacking in certain features, such as the cross-correlation with Focal Adhesions in FAFCK [39]. Integrating the cross-correlation with FAs will enhance the quality of SPOCC. SPOCC should be expanded to quantify other types of cytoskeletal structures such as microtubules and intermediate filaments. In such works, it is important to keep in mind that other type of filaments mostly have a lower persistence length and so be curvier than stress fibers, which will further aggravate the complication arising from breaking up the filaments in straight lines. This underscores the requirement for a fiber stitching algorithm even more.

The paper claims the unique integrability of SPOCC with other biophysical techniques, such as migration and would healing assays, but no such work has been shown in the paper, excepting the correlation with AFM. Though the suggestion is extremely fascinating, further studies are required to actually correlate SPOCC results with such assays, which will uncover more information regarding how SPOCC can be modified to improve the integrability. But it is worth mentioning that wound healing and such assays take a long time to perform and provide an average characteristic of the cells in that period, which renders them useless for fatter biological processes; if the cytoskeletal information can indeed be correlated with biological characteristics such as motility, SPOCC, owing to its real-time measurements, can bridge a gap that is dearly needed in biology.

The correlation of stress fiber pattern and the corresponding cell elasticity (from AFM force curve measurements) is mentioned in the paper, but no empirical model is provided as to how the stress fiber reorganization from nest-like to semi-parallel is likely to increase the Young's modulus of the cell, essentially rendering the cell stiffer. It is likely that different types of stress fibers have different elastic properties and hence contribute differently to the overall stiffness of the cell. It would be fascinating to calculate the tension being carried by the fibers based on their types and locations. Such experiments are required to verify and confirm such effects before SPOCC measurements can be truly correlated with AFM data.

The biological conclusions drawn from SPOCC analysis are supported using known biological assays, such as Western Blot analysis. The identification of a partial EMT cytoskeletal phenotype and compartmentalizing EMT into two steps (formation of fibers and alignment of fibers) are indeed novel. But it is essential to obtain a better understanding of the genetic cascade responsible for the process which is lacking in the paper. Multiple pathways have already been reported for EMT and cytoskeletal rearrangement in cells. This paper suggests that formation and alignment of stress fibers are two distinct steps and as such should have different genes responsible for their occurrences. Noteworthy here is the impact of temporal cascading of these signals; the fiber alignment signaling has to be activated after the fiber formation signaling. Tracking the RNA/protein expression levels during EMT is the most direct way to answer these questions, but that can be time-consuming and expensive. Previous reports of RNA-seq based EMT mapping and identification of intermediate states [40] should be used instead. By measuring the RNA/ protein levels of the partial EMT phenotype, one can determine which one of the previously reported intermediate states this phenotype corresponds to and where it fits into the overall genetic cascade. This will shine further light on which signals are responsible for fiber formation and alignment. It will be illuminating to carry out these analyses on a larger number of cells to enhance the statistical validity of the results. Finally, including different cell lines and cancer types in such studies will aid in understanding how universal these phenomena are.

Overall, the SPOCC technique is a robust and novel image analysis tool that can provide more quantifiable biological

information than comparable techniques. But further studies are definitely required to verify the integrative nature of SPOCC as well as its importance in biology. In reality, SPOCC is a technique with a huge potential that can serve as a building platform for more sophisticated and comprehensive studies.

Conflict of Interest

None declared.

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