Measuring the average cell size in cellular tissues using Fourier Transform

Tess Homan, Sylvain Monnier, Cécile Jebane, and Hélène Delanoë-Ayari

Biophysique, ILM, Université Claude Bernard Lyon 1, Villeurbanne, France

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We present an in-depth investigation of a fully automated Fourier-based analysis to determine ensemble averages of the cell size in 3D biological tissues. The results are thoroughly tested using generated images, and we offer valuable criteria for image acquisition settings to optimize accuracy. Cells cannot be imaged indefinitely either because of movements, or photo-bleaching. It is important to know on what to spend properly the limited imaging time, i.e. choose between a higher x, y-resolution or a larger field of view, a larger z-stack or a smaller z-step. We demonstrate that the most important parameter is the number of cells in the field of view, but we show that accurate measurements can still be made on volume only containing 3x3x3 cells. The resolution in z is also not so important as long as there is at least one pixel per cell. We validated our tool on real in vitro images of cell aggregates. The technique appears to be a very promising tool for very fast live local volume measurement in 3D tissues in vivo.

Volume is a key parameter in various fundamental biological processes such as cell growth, division or fate [1][2]. It is tightly regulated during cell cycle [1] and is dependent on the cellular microenvironnement chemical and physical properties [2][3]. It can be regulated by cellular tension [4]. Different techniques have been used for measuring isolated cells volume such as 3D cell reconstruction [2]. Fluorescence Exclusion measurements [5] and commercial coulter counter [6]. The regulation of cellular volume within 2D/3D tissues is unknown so far, mainly due to technical limitations.

Indeed, obtaining cell area/volume necessitates precise cell segmentation of its boundaries in 3D, which will most often require the acquisition of a z-stack of a sample in which the membrane [7] or the intercellular space [8] have been made fluorescent. However, the imaging of living samples is restricted by cell movements, photobleaching or even cell death (photo-toxicity). Capturing an entire z-stack can take up to several minutes at high resolution, which is on the same timescale as the rearrangement of cells inside the tissue. In addition, cells are very sensitive to light, with overexposure leading to cell damage. The amount of images which can be acquired to create a z-stack is therefore limited by the amount of light cells can endure. So developing a new tool which would require the minimum z slices acquisition is of real importance for time follow up of in vivo tissues.

Based on previous work [9], we propose here a 3D Fourier transform tool for live measurement of cell volume within 3D tissues. Fourier analysis describes the image as a superposition of sinusoidal functions (repetitive patterns with a set frequency) [10]. Most images are a combination of many frequencies, but cellular tissues are made up of distinct units: cells. Therefore the result of the Fourier analysis has one dominant frequency corresponding to the average cell size. We tested here how accurate this method can be by using in silico simulated data of 3D tissues. This also allowed us to independently vary all important parameters, such as the number of cells in field of view, cell size homogeneity, and acquisition resolution.

This paper starts by describing how the artificial and real cell images are made, followed by a short explanation of the Fourier Transform protocol. Subsequently we investigate the influence of several variables: number of cells in the field of view, pixel size in the z direction, thickness of the z-stack. Finally, we assemble protocals for image acquisition parameters both in a set of rules, and in straightforward look-up graphs. we finally show results on living cellular aggregates, which represent good models of in vitro tumours [11].

I. MATERIAL AND METHODS

A. Cellular aggregates preparation

We use HT29 cells for these experiments. Cells are culture in DMEM Medium (GIBCO 61958-026) supplemented with 10% FBS (Pan Biotech P308500) and 1% Penicylin-Streptavidin (GIBCO,15140-122). They are maintained at 37°C with 5% CO₂ and passaged twice a week. Aggregates are formed using Ultra Low Adhesion 96 Well Plates (Greiner bio-one, 650970). After passaging, cells are counted and diluted to a concentration of 10 000 cells/mL. From this stock solution, a desired number of cell is subsequently seeded into a well (for example 300 cells/mL) and left to grow into aggregates for a minimum of 48h. To image the aggregates, we use a 40 µm-high microfluidic channel. This prevents aggregates from moving around and simplifies media changes. The aggregates are injected inside the microfluidic chamber and we wait for 1h before adding FITC-Dextran and CO₂ independent medium (GIBCO 18045-088), to let the aggregate relax.

helene.ayari@univ-lyon1.fr
FIG. 1. (a) Typical z-slice of an HT29 aggregate using two-photon acquisition (b) One z-slice (128 × 128 pixels) out of the 3D stack with cells generated using the Voronoi method. The cells have an average size of 11 pixels. Note that faces parallel to the z-slice can result in large white areas, (c) A 3D rendering is made of the entire z-stack where dz is equal to 1 pixel i.e. the same resolution in x, y, and z, (d) Fourier transform from the image displayed in (b) the 3D matrix in Fourier space after an FFT shift is displayed as a 3D image. A bright center pixel and a light spherical shell are visible. A 2D cross section is shown in (e). (f) Spectral density energy along the purple and green dashed line displayed in (e), in blue is the radial average of the spectral density, enabling a very accurate measurement of the peak which corresponds to the main frequency (and thus cell size) found in the original image.

B. Two-photon imaging of cellular aggregates

We use two-photon setup on a Nikon microscope equipped with a 780 nm laser at the nanoptec facility in Lyon and a water immersion 40X objective (NA=1.15). The extracellular space was stained with FITC-Dextran diluted at 2 mg/ml in the C02 independent cell culture medium (GIBCO 18045-088). All experiments were conducted at 37°.

C. Artificial cell images generation

For testing our Fourier Transform algorithm, we generate synthetic 3D images of control properties mimicking real tissues (Figure 1b). Images are made of $L \times L \times L$ pixels. Random points (representing the centers of the cells) are added automatically one after the other in a 3D matrix. To each point a radius is randomly taken from a normal distribution centered on $r_0$ with a width $\sigma$. A new point, $i$, is only added if it’s sphere with radius $r(i)$ fits in with all other spheres, if not, a new random point is chosen. Above a certain threshold it will be impossible to add new cell centers because there is no more space. We use a maximum number of attempts to add a cell as a cutoff. Note that at that point we still do not have a regular lattice, even if we set $\sigma$ equal to 0. The width of the size distribution can be adjusted in two ways, either by changing $\sigma$ or by changing the number of possible attempts for filling the lattice. $r_0$ defines the typical number of pixels per cell. Changing this value enable to simulate different magnifications used when acquiring real data with different objectives at different resolution. The total number of cells that are visible in the image can be set by changing $L$, which would correspond to
changing the field of view in data acquisition. To avoid edge effects the actual space in which we distribute the centers runs from $-2r_{\text{max}}$ to $L + 2r_{\text{max}}$ in $x$, $y$, and $z$-direction. The images are then cut out from the center to create an $L \times L \times L$ z-stack.

We end up with a distribution of cell centers, but in experimental data it is the cell boundary that is imaged. To generate the faces that separate the cells a Voronoi tessellation is used. In 2D, a serie of cell centers, or points, can be divided into cells using lines that run right in the middle between two centers. This results in a surface filled with polygons, one polygon per center. This also works in 3 dimensional space, only instead of edges this time the result of the Voronoi tessellation describes the faces that separate neighboring cells. One set of cell centers leads to one unique set of edges or faces. Therefore, by fixing the center points, the volume and size of each cell is also imposed. For each pixel the distance to all cell centers is determined and the two smallest distances are compared. If the two closest cell centers are equally far away from the pixel we are examining, then the pixel lies on a Voronoi face. Because this system is only pixel accurate we use a threshold; if the difference in distances to the closest two points is smaller than the threshold, this pixel is part of the boundary separating the two cells. By adjusting this threshold value, the thickness of the edge can be set to match the experiments.

The pixels on the Voronoi boundaries can be made 1 (to create a black and white image), or can be scaled with the relative distance to the two closest points, creating a smoother boundary, which is what we use here as it gives images much closer to real data (Figure 1(b)). Subsequently, for every $z$-value an image is saved, this creates a z-stack similar to the experimental results. Using a 3D software (Amira) the entire image stack is segmented and visualized in figure 1(c).

We mainly used two batches of artificial images. In both cases, $L = 128$ pixels. The first one was created to test the influence of cell size for a given field of view $L$, $\sigma$ was set to 0, and $r_0$ was varied between 1 and 10 by step of 1. In the second one, the radius was fixed to $r_0 = 5$, and $\sigma$ was varied between 0 and 2 by step of 0.2 so as to affect the width in size of the cell sizes distribution.

D. Fourier Transform Analysis

Before applying Fourier Transform, we first decompose the images in periodic ($p$), plus smooth components ($s$), and apply the transform on $p$ to avoid edge effects artefacts. For discrete signals, such as our 2D and 3D images, a Discrete Fourier Transform (DFT) is computed using a Fast Fourier Transform (FFT) algorithm. The Fourier transform ($X$) of a 3D image ($x$) of size $(N \times M \times L$ pixels) is calculated using

$$X(s+1, t+1, u+1) = \sum_{n=0}^{N-1} \sum_{m=0}^{M-1} \sum_{l=0}^{L-1} e^{-\frac{12\pi i n}{N}} e^{-\frac{12\pi i m}{M}} e^{-\frac{12\pi i l}{L}} x(n+1,m+1,l+1).$$

Using FFT, we calculate the spectral density energy given by $|X(s,t,u)|^2$. The result is a 3D matrix and after a 1 shift the distance to the center (brightest pixel in the middle of the image stack) corresponds to the radial frequency. The higher the value of the element in the matrix, the more present is this particular frequency. We thus expect to have peaks located at the average distance between cell centers $f_{DF}$. In the case of a confluent tissue (which is what we simulate in this study), we expect that $f_{DF}$ gives an accurate estimation of the the average cell diameter. When displaying the Fourier results as a 3D image, we indeed see a bright shell around an even brighter center pixel, see figure 1(d). The average size of the cells is now simply one over the distance of this shell to the center. In figure 1(e) a horizontal slice of the Fourier transform matrix is shown. The bright ring is easily visible. The intensity of the pixels lying on one line through the center of the matrix is given in figure 1(f). The magnitude of the center peak is too large to display and was set to zero, but from this data it is clearly possible to measure the distance from the two strongest side peaks to the center. This is the data from only one line of pixels through the image, but the results will be similar under every angle as cell dimensions are relatively homogeneous in $x, y$ and $z$. To properly determine $f_{DF}$, we take advantages of the spherical symetries of the FFT, and interpolate the results in spherical coordinates ($f_r$, $\theta$, $\phi$), then we calculate the energy in a shell of thickness $df$, summing over $\theta$ and $\phi$ and obtain $e_{f_r}$, that we normalize by the total energy in the signal and get $e_n$ that we then plot as a function of $f_r$ (Fig. 1(f), blue line). The integral of $e_n$ underneath this curve is therefore one by definition and we show here that the maximum of this function leads us to a very good estimate of the average cell size in the tissue.

II. RESULTS

A. Cell sizes

We first tested the accuracy of the FFT method for calculating cell size. We compare the cell size given by the peak of the Normalized Fourier energy density and the average cell size directly obtained in real space on the artificial images. However, even though for the original data, we have the exact locations of all cell walls, it is still not trivial to define an average cell size. From the Voronoi tessellation we easily get the volume of each cell (completely enclosed in the image), whose average value will be noted $V$, but from the Fourier Transform
peak location we get a frequency, which will lead to a diameter that we note \( D_F \). Coming from the cell volume it is obviously possible to also calculate an average cell diameter, but an assumption on cell shape has to be made. Choosing a cell shape between spherical and cubical can alter the volume calculation by 30%. But as cells completely pave the space (no holes in the tissue), it appears quite natural to assume a cuboidal shape and get an average size \( D_{V_{or}} = V^{1/3} \). We will more exactly use \( D_{V_{or}} = V^{1/3} - 1 \) (pixels), as in the cell volume calculation the contour is always included, and for the distance between two cells it would count twice.

In figure 2, the analysis results for several values of \( r \) is shown. The peak becomes both wider and lower with increasing cell size. For each cell size, we can easily determine the peak location and deduce \( D_f \). We show here the result of \( D_F \) as a function of \( D_{V_{or}} \) (Fig. 2b), it is very well fitted by a line of slope 0.96, \( r^2 = 0.992 \). The FFT method is thus well designed to get the average cell size. We will now study the influence of different parameters on the error on the measurement, defined as \( D_f - D_{V_{or}} \).

### B. Effect of different parameters on cell size measurements.

For all the results that follow, we now used a set of 11 images, which have been generated with \( r_0 = 5 \) (i.e. an average cell size \( D_{V_{or}} = 10 \) pixels)

#### 1. Effect of the number of cells per field of view.

From the point of view of the Fourier transform, the most important parameter is the number of cells in field of view, in both \( x \), \( y \), and \( z \), far above the number of pixel per cell, as we already demonstrated in 2D [9]. The higher the number of visible wavelengths, the stronger the peak at that corresponding frequency in Fourier space (see Fig. 3b). We tested here what is the minimal number of cells in a given volume, to get an accurate result, this may be important if one wants to make local measurements of cell volume.

We plotted the mean error on cell size measurement, as a function of the size \( L \) of the image (taken the same in \( x \), \( y \) and \( z \)) (see Fig. 3b). We see that even when \( L = 32 \), the results stay relatively good with a mean error which stays zero, and a standard deviation around 3%. As cell size was around 10 pixels, this means that we need at least 3 cells in each direction in the image to obtain a valid result. This opens the way for very local measurement of cell sizes.

#### 2. Effect of the pixel size in \( z \).

From an image acquisition perspective, one of the hardest things to obtain is a high resolution in \( z \)-axis direction. So far our resolution in \( x \), \( y \), and \( z \) was identical, but often the distance between layers, \( dz \), is twice, or even four times the resolution in \( x \) and \( y \), \( dx \) and \( dy \). How does this influence the measurements?

Coming from the generated data, these are very easy parameters to check. We simply start with an image of \( 128 \times 128 \times 128 \) pixels, and then start deleting information, only keeping one slice every \( dz \). \( dz \) is thus representing the pixelsize in \( z \). In figure 4, we see the \( dz \) influence on the Fourier curves. As long as the resolution is set below cell size, the cell size measurement stays correct. As expected, if the resolution in \( z \) becomes lower than one cell size the results are biased, and we obtain cell sizes bigger than expected with a larger error. This results...
are really encouraging as it means that one do not need extreme performant microscopy in term of z resolution to get an accurate cell size measurement.

3. Effect of sample thickness.

Finally, the total number of layers is often significantly lower than the total number of pixels in x and y direction. We could also wonder if 3D is really necessary, can we not just use a single cross section? Results to this question can be found on figure 5. We see that as the number of slices decreases the results are more and more biased, the cells appear smaller than they really are (which is coherent as when using only one plane, we will have a lot of "cut" cells appearing in the field of view, giving the impression that cells are smaller than they really are). We also recover the results from II B 1 : we need around 30 slices to get a proper measurement which corresponds to around three cells. Now, can we combine the results from II B 2 with those last ones? In our case would it be possible to take only 3 cells in z (32 slices) and dz = 10, i.e end up with a stack of only 4 images? Result of such configuration is shown in red on Fig. 5b. The measurement is really good even if there is a slight increase in the standard deviation on the measurement.

III. RESULTS ON TWO-PHOTON STACKS OF CELLULAR AGGREGATES.

After testing thoroughly our algorithm “in silico”, we now measure its performance on real data. For that, we formed cellular aggregates of HT29 cells imaged using two-photon microscopy, and a fluorophore which only penetrates in the extracellular space (Fig. 6). We computed our energy density function (a few seconds of computation), but we then needed to compare it to “real” cell size measurement. We then segmented 100 cells in our dataset manually using Amira software (a few hours of segmentation). The excellent adequation of these two measurements are shown on Fig. 6. The mean value of the manually segmented cell is 11.4 ± 0.2 whereas the position of the peak of the Fourier Transform gives us 11.4 ± 0.7.

IV. CONCLUSION.

We present here an original method based on Fourier Transform to calculate cell size very accurately and precisely. We tested thoroughly the accuracy of this new tool, which gave us very interesting results, as the method can be applied very locally on a volume of 3*3*3 cells, and do not necessitate a very high accuracy in z resolution. It is interesting to note there that one single set of data can
be used several times with different sectioning (varying dz for example) to get different measurements of the peak location, the results could be averaged and lead to a potentially very accurate final result. There is no doubt that this technique can be applied also in vivo in situations where automatic cell segmentation is too difficult as in the data presented here. Further development could enclose the study of cell anisotropy, this would necessitate fitting data by an ellipse instead of a sphere.

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