Investigating the Porosity of Agarose Hydrogel through Single Fluorescent Particle Tracking via Confocal Microscopy

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ABSTRACT

Agarose hydrogel, a hydrophilic polysaccharide, has become a quintessential polymer for the study of biological physical phenomena. Its structural similarity to living tissue and extracellular matrix fluid facilitates its application to new biomedical technology. While there are several techniques for understanding it at a nanometer scale, high production costs of hydrogels and sophisticated experimental setups limit further developments, and thus, its commercialization. In this work, a novel technique applying a standard epifluorescence confocal microscope to single particle tracking along with a culminating laser scanner was used to determine the trajectory of quantum dots diffusing through agarose hydrogel in three-dimensional space. In particular, we utilized an extremum seeking algorithm that automatically moves towards the peak of the measured intensity within the point spread function and, as a consequence, to the vicinity of the source particle. By plotting the paths of the diffusing quantum dots, which produced highly concentrated clusters where pores in the agarose were located, a visual representation of the structure of the hydrogel was produced. Initial results provide a promising wealth of information related to the size, shape, and volume of the pores that later studies may use to further develop our understanding of the porosity of agarose hydrogel. Though an estimation of the size could be done through a spherical fitting algorithm, further analysis suggests an ellipsoidal fitting algorithm to be more practical. Such findings will be utilized in future scientific research to better advance the use and understand the effects of agarose hydrogels in biomedical engineering and industry, which might otherwise have been distorted by a lack of understanding of agarose hydrogel porosity.

INTRODUCTION

Often used to encapsulate pharmaceuticals and simulate tissue, agarose hydrogel ($C_{24}H_{38}O_{19}$) has undeniable utility and potential to biomedical engineering. The gel is a valuable component of many new technologies produced, from fields ranging from food sciences to pharmacology to tissue engineering to genetics[2]. However, our lack of understanding of its structure, in particular the nature of the pores within it, greatly undermines the accuracy of research that utilizes agarose hydrogel, because to separate the effects of a treatment from the impact of the pores of the agarose hydrogel is nearly impossible. A thorough investigation of the structure of agarose with resolution on the scale of nanometers could potentially have significant impact on projects and industries that utilize agarose hydrogels.

Agarose is also a commonly used gel in electrophoresis, and its prevalent use can be linked to its strength and porosity. The internal structure of agarose hydrogel may be thought of as built with a large number of rigid chains, arranged such that there may be larger gaps of empty spaces, which we call pores[4]. The size, shape, and arrangement of these pores affect many physical and chemical properties of the agarose; for example, the porosity determines how much of a specific drug may be carried by the hydrogel, or how much it will flex or contract in mechanical applications[3].

Figure 1. The agarose used for this investigation in liquid (top right), gel (bottom left), and powder (bottom right) form

TRACKING ALGORITHM

To properly investigate the structure of hydrogel, we tracked the path of particles traveling in the matrix of the hydrogel so as to not damage or distort the internal structure of the gel, using Dr. Trevor Ashley's adaptation of confocal microscopy to quantum dot tracking. Ordinarily, confocal microscopy is applied to larger structures like cells; however, Ashley adapted it for single particle tracking by implementing an algorithm that seeks the extremum of the pattern of intensity, or point spread function, by use of an avalanche photodiode[1]. Because the particle is located at the peak of the intensity, by pursuing the point of maximum intensity, Dr. Ashley's algorithm can locate and track a single particle diffusing through a medium.

$$\dot{x}_s = R(\omega_1 \sin\theta\cos\phi + \omega_2\cos\theta\sin\phi),$$
$$\dot{y}_s = -R(\omega_1 \sin\theta\sin\phi - \omega_2\cos\theta\cos\phi),$$
$$\dot{z}_s = \omega_1 R\cos\theta,$$
$$\dot{\theta} = \omega_1\left(1 - K_P\frac{dI}{dt}\right), \quad \dot{\phi} = \omega_2,$$

Figure 2. The single particle tracking algorithm

In this algorithm, I is the intensity measurement at time t. The user may adjust R (the radial distance between particle and the volume of sample that may be efficiently detected through the microscope), K_p (the rate of convergence to the sphere), and ω_1 and ω_2 (rates of oscillation of the focal volume[1].

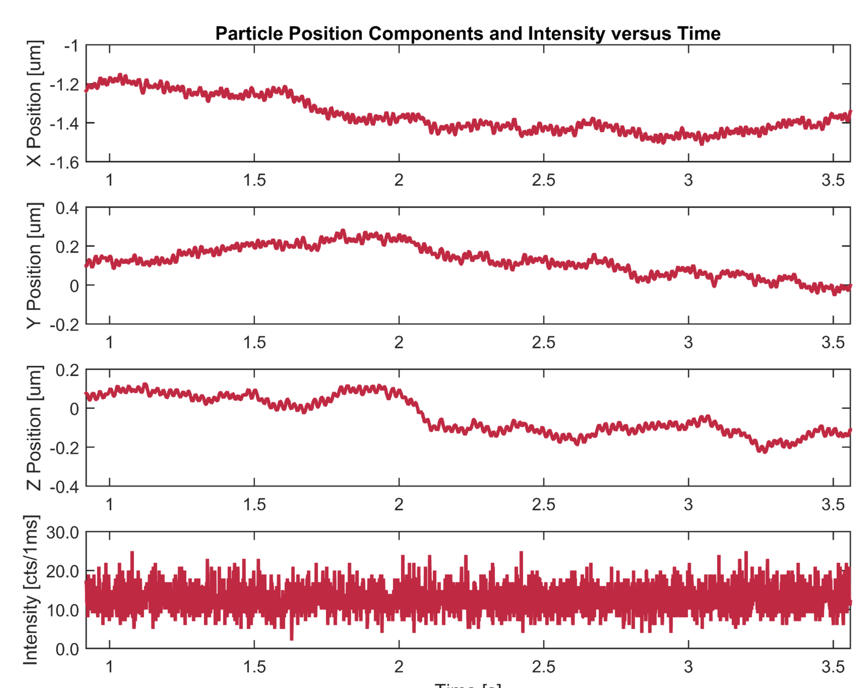

Figure 3. A graphical representation of a successful tracking run utilizing the algorithm. The particle components are mapped in the first three graphs and the intensity in the last. The 3-D model of this run can be found in Figure 5.

METHODS

In order to prepare the hydrogel sample for single particle tracking, 1 microliter of quantum dot solution, diluted with water to a volumetric ratio of 1:500, was pipetted onto a coverslip. To this, a heated mixture of glycerol, in order to slow the movements of the quantum dots within the agarose matrix, and 3.6% concentration of agarose hydrogel was added and manually stirred into the quantum dot solution. A smaller coverslip was placed onto the mixture and glued with a clear sealant. The slides were allowed to return to room temperature before being placed into the refrigerator to further cool, and thereby slow the quantum dots for optimal tracking. The cooled slides were mounted on a holder, which was placed on the stage of the microscope.

The general location of a single quantum dot was manually acquired, upon which Dr. Ashley's single particle tracking method was used to more precisely identify, predict, and follow the trajectory of the particle's path. The algorithm was implemented in LabVIEW on a National Instruments compact Reconfigurable Input Output (cRIO) system using a sample rate of 1 kHz.

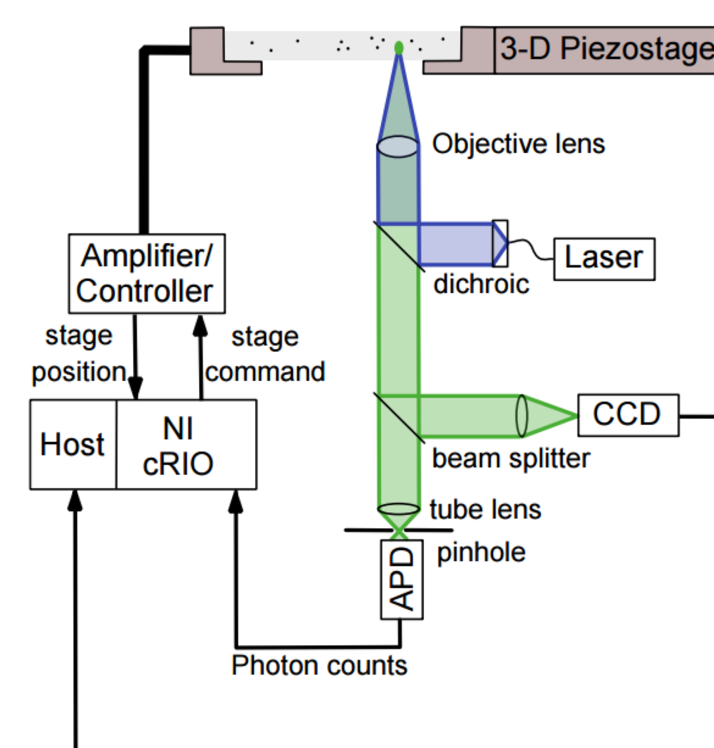

Figure 4. A diagram of the single particle tracking apparatus and equipment. Light from the sample passes through the dichroic mirror and is split into a 30:70 ratio by the beam splitter, the smaller portion of which is sent to the CCD camera, which displays an image on the computer screen. The other 70% is sent to the APD (avalanche photodiode), which detects emitted photons

The acquired data was analyzed in MATLAB. First, the measurements were plotted both as time series and as 3-D plots. We then manually excised the start and end indices of each pore by adjusting the start and end parameters of the algorithm to fit each concentrated cluster of data points. Additionally, we wrote an excising data function in order to store the start and stop indices of each data file, so we could easily use the results of this manual procedure to extract only the portions of the trajectory that appeared to be within a single pore rather than moving between pores. To investigate the volume of the pores, we found the values for the semi-principal axes, a, b, c to fit the formula for volume of an ellipsoid: V=$\frac{4}{3}\pi abc$. Consequently, we created a principal component analysis function in MATLAB, that reorients the initial semi-principal axes of the ellipsoid to fit the largest possible variance, which then resulted in normalizing the data. By using the eigenvalues of the covariance matrix of the normalized position data, the function then created a new matrix of coordinates to adjust for the linear transformation. where we then used to find the maximum range for each of the adjusted x, y, z to calculate the length of the three semi-principal axes.

RESULTS

We collected over a thousand runs of data, however after analyzing and excising the data, approximately four hundred runs of raw data remain. The following plots depict the position of the particle as it moved in three dimensional space from an excised portion of one of our successful tracking runs.

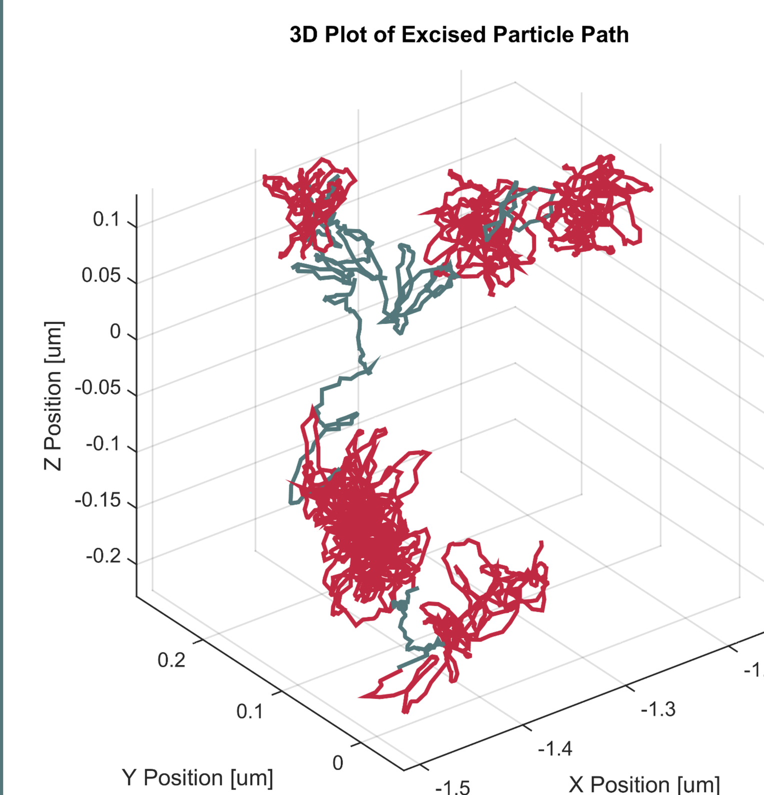

Figure 5. An example of a 3-D plot of a raw data file, prior to excising segments inside individual pores. The particle began in the top before making its way towards the bottom. Each distinguished individual pore in which the particle entered is colored red.

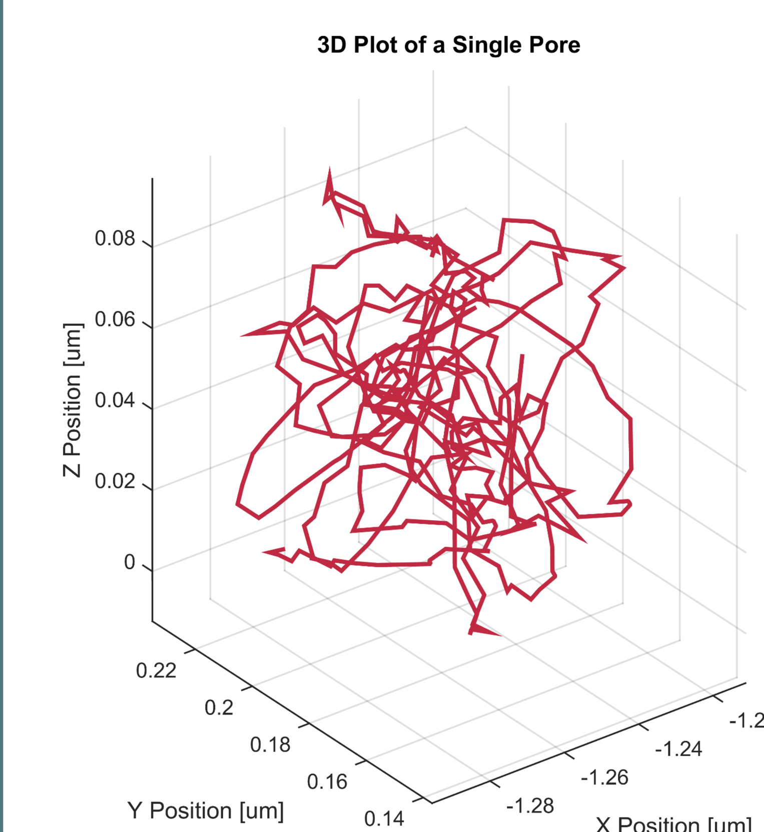

Figure 6. A 3-D plot of a single pore from the data displayed in Figure 5. The red lines depict the movement of the particle as it was contained in the pore membrane. The shape appears ellipsoidal, however also seems to closely resemble a sphere.

DISCUSSION

While the equipment prototype is prone to error due to nanometer level analysis and the rather novel approach to the study of agarose hydrogel porosity, the consistency in intensity levels along with subtle movements in x, y, and z position justify the success of the run (Figure 3). Note that significant changes in either position or intensity would resolve itself to the complication of the program tracking two different particles under single particle tracking run. Additionally, through analyzing the excised plot, we determined that the concentrated clusters suggest pores of the hydrogel, as the particle moves in a manner contained by the ellipsoidal membrane. The thinner lines result from the seeking of a new pore after the particle leaves its previous one.

On a set of 24 data runs, our data indicates that for an ellipsoidal approximation given by $\frac{x^2}{a^2}+\frac{y^2}{b^2}+\frac{z^2}{c^2}=1$, the average a, b, and c values for the semi-principal axes are 6.023×10^{-2} μm, 4.831×10^{-2} μm and 3.722×10^{-2} μm with a standard deviation of .0153, .0098 and .00753 respectively. The smallest volume of a single pore was 1×10^{-4} μm^3 and the largest was 6.68×10^{-4} μm^3. We also discovered an outlier, with a volume of 1.871×10^{-3} μm^3 ,yet found it to be most likely two juxtaposed pores within the agarose, given that it's a value is nearly double the a values of the other pores. The mean volume of the pores, excluding the outlier, was 4.338×10^{-4} μm^3, with a standard deviation of 1.588 × 10^{-4}

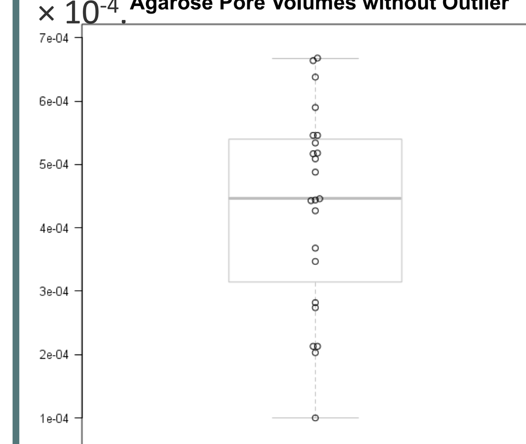

Figure 7: A dot plot with a superimposed box plot of the 23 volumes, excluding the outlier, of the approximate volumes of the pore. Each circle represents one pore.

Because we investigated only the raw data, we can only make preliminary analysis on the porosity of agarose. However, confocal microscopy is a method that is extremely unlikely to damage the internal structure of the agarose, as opposed to other methods that might warp the arrangement of the pores. Nevertheless, a more accurate analysis could be done through translating the measurements of confocal volume positions into particle positions. Additionally, a method or algorithm to automate the process of identifying pores is much needed; for example, one potential method involves recognizing when the axes of the ellipsoid suddenly changes dramatically, in either direction or length, thereby indicating that the particle has left the pore. Though our ellipsoidal approximation provides a competent model of the shape of pores, a more precise approximation of the shape of a pore could still be developed. Continuous analysis of the arrangement of the pores and understanding its flex and contraction could potentially be done to enhance understanding about the nature of the pores. In addition, further experiments that utilize water as a medium, as opposed to glycerol, may be performed to confirm our results, as water is conventionally the medium in which agarose hydrogel is used in biomedical applications.

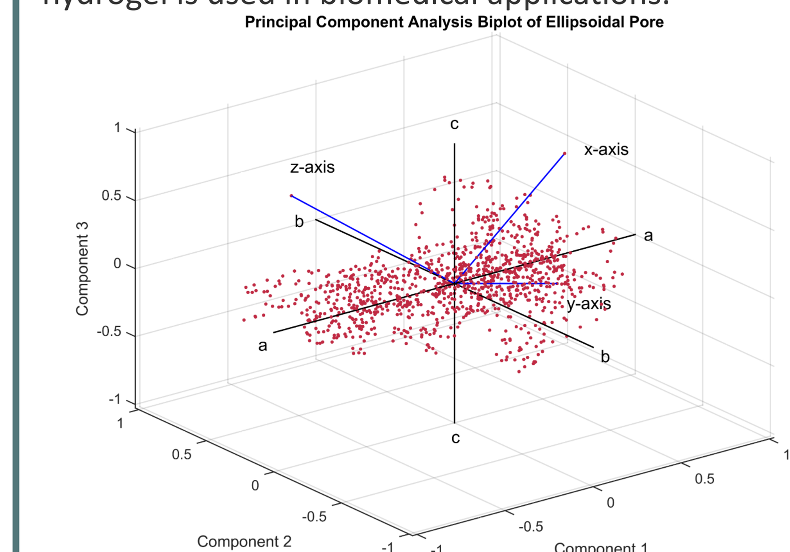

Figure 8. A biplot of the pore in Figure 6. Individual dots represent the location of the particle at a certain time given a sample rate of 1 kHz. The original x, y, z axis are shown in blue, and the new axis of the normalized principal component coefficients following PCA are shown in black.

CONCLUSION

On the whole, our project has produced valuable insight into the porosity of agarose hydrogel through not only understanding the shape of the pores, but also their orientation and volume. Consequently, through this preliminary study, we have also demonstrated that the pores in agarose hydrogel are on average around 4.338×10^{-4} μm^3 in volume with an ellipsoidal shape, with many pores more closely resembling spheres. It is important to note that our project is one of the first, in the status quo, to apply single particle tracking using a confocal microscope to the study of the porosity of agarose hydrogel. Thus, as one of the first of its kind to accumulate a large amount of data using a single particle tracking method, our research project investigates agarose hydrogel from a perspective that has yet to be fully explored by modern science. As such, our investigation paves the way for future investigations of the substance that is ubiquitous across the biotechnology field, yet not sufficiently studied. Once our understanding of agarose hydrogel is complete, we can greatly expand the depth of research in biotechnology beyond its current barriers.

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