Study on Measurement Method of Three-dimensional Position of Unlabeled Microspheres under Bright Background

Ke Xu

xksky1234@163.com

Shenyang Jianzhu University

Bingge Wang

Shenyang Jianzhu University

Research Article

Keywords: Three-dimensional position of the microsphere, off-plane stigmatic imaging, myocardial cell, single particle tracking

Posted Date: April 11th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2788805/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Current Nanoscience on September 15th, 2023. See the published version at https://doi.org/10.2174/1573413719666230915103459.

Abstract

Using computer vision technology to obtain the position and trajectory data of particle probe microspheres from microscope images has important significance and value in the molecular field. However, most of the existing microsphere measurement methods are based on transmission, which can only be measured under transparent samples and substrates, are not suitable for the application scenario of living cell measurement. In this paper, a method based on reflectivity imaging is proposed to measure the three-dimensional position of the dark microspheres in the bright field. Based on the outermost ring radius method, the relationship between the inner ring radius of the microsphere spot and the out-of-focus distance was explored to measure the coordinates in the Z direction. Cardiomyocytes were combined with 10um size silica microspheres. Experiments show that in a bright field with high perturbation environment, it can achieve high precision measurement of dark microspheres and achieve three-dimensional position measurement with accuracy of 50nm in XY direction and 100nm in Z direction.

Introduction

With the wide application of microscope digital imaging systems, multi-target tracking technology has been gradually applied to many fields, and the study of dynamic processes at the molecular level has gradually become possible. Obtaining data such as cell position and motion trajectory from microscope images has important significance and value in medical diagnosis and treatment, drug research and development (Neutsch et al. 2020). However, the original data of microscopic images often have problems such as large amount of data information, time-consuming and labor-intensive manual processing. If computer vision technology can be used to achieve accurate target detection and tracking of microscopic images, and even exceed the judgment accuracy of some professional doctors, it will greatly improve the efficiency of biological research, so that human beings can explore more mysteries of the microscopic world.

The microspheres can be well connected with the measured cells and do not affect the cell's own activity, mobility and ability to interact with the environment. In addition, only one diameter parameter is needed to describe the model of the microspheres, and the optical imaging has the characteristics of radial symmetry (Mykhailo et al. 2023), which has strong photostability, and is very suitable for the research of non-damage three-dimensional position measurement as a particle probe or carrier. Most of the existing microsphere measurements are based on transmission and can only measure transparent samples and transparent substrates, which are not suitable for the application scenario of live cell measurements. When the microsphere is placed in the sample pool with cells, the cell itself will also produce interference or diffraction fringes, which will undoubtedly increase the difficulty of measurement. Therefore, a computer image processing system that can distinguish the microsphere spot from the sample and other interferers spot is urgently needed, and the measurement accuracy can reach nanometer or even sub nanometer level. In turn, it provides comprehensive motion parameters for the control and drive of life-like robots, and ultimately provides help for subsequent medical analysis.

Traditional methods cannot quantitatively study the mechanism of action of single biomolecules, while single-particle three-dimensional tracking technology has become a key technical means to understand the molecular dynamics in vivo because it can capture the spatiotemporal behavior of subcellular (Park et al. 2019). The existing three-dimensional positioning technology of microspheres can only achieve high-precision local size measurement, which is difficult to adapt to the requirements of three-dimensional positioning of microspheres in the field of single molecular dynamics. Other methods also have shortcomings in scope of application and speed (Wu et al. 2020).

Using computer technology to process two-dimensional video or image to obtain three-dimensional coordinates of microspheres in three-dimensional space is a very effective method (Huang et al. 2020). At present, in the three-dimensional positioning and tracking of microspheres, there are many mature methods that can be used to measure the motion of microspheres of various sizes. It mainly includes two aspects: transverse measurement and axial measurement. The measurement technology of lateral measurement has been relatively mature, such as centroid method, Hough transform method (Tang et al. 2021), Gaussian fitting method, gradient method based on radial symmetry, quadrant interpolation method based on improved one-dimensional cross correlation, etc. For high-resolution positioning in the axial direction perpendicular to the optical axis, there are measurement methods based on matching known experimental images, such as outermost ring radius method (Zhao et al. 2022), cross-correlation method, vector projection method, etc.

The above measurement methods are all described based on transmitted particles. Reflective microsphere imaging is still in a relatively blank state at present. The measurement method based on transmission uses light field to form interference fringes through the microsphere and matrix, and then analyzes the movement and change of the microsphere, which can only measure transparent samples and transparent substrates, which greatly limits the measurement range and application scenarios (Leister et al. 2021). In this paper, an unmarked measurement method for bright-field imaging based on reflective microspheres is proposed. By changing the traditional digital holographic imaging using Fresnel diffraction law (Zeng et al. 2022), when the microspheres are further reduced, the more accurate Rayleigh Summerfield function is used to reconstruct the three-dimensional measured microspheres, and an improved method based on Hough transform method and cross-correlation method is proposed. Firstly, the center of the microsphere was roughly located, and then the radial contour was formed by drawing the light intensity change curve in the rectilinear direction through the coarse positioning center. In the radial contour drawn from different central coordinate points, the phase of the same ring in different directions was found, and the coordinate of the point with the minimum phase variance was the central coordinate of the microsphere. Combined with the sub-pixel theory (Wang et al. 2022), the measurement accuracy is further improved. The existing outermost ring radius method is improved. Because the measurement is carried out in a bright field in a liquid environment, the microsphere diffraction spot and background noise are blurred, and it is difficult to distinguish the position of the outermost ring. This method has no restrictions on the material of the microsphere itself, and the substrate no longer needs to be the same material as the microsphere. It is non-cytotoxic and can be used to measure the dynamic beat trajectory, beat propagation and conduction velocity of the

myocardial cell monolayer for a long time (Dou et al. 2022). For microspheres with different materials and substrates, it can not only be applied to cell measurement, but also is expected to be combined with other measurement methods to further improve the measurement accuracy and measurement range of existing measurement methods.

Three-dimensional position measurement

XY direction position measurement

When the spot position is measured in the background of bright field in living cells, the cell motion interferes with the detection of microspheres. At the same time, the reflection effect produced by the background also has a negative impact on the detection results, so the spot detection operator should have strong anti-interference performance. In addition, it is necessary to ensure the accuracy of the spot when multiple spots in the same area are close to each other. In order to solve the above problems, overcome the noise and uneven light intensity. In this section, based on the measurement accuracy and applicability of the reflected microscopic image of liquid environment, a spot center location algorithm suitable for bright field is proposed, which is based on the improved method of Hough transform method and cross-correlation method. Firstly, the image of microspheres was preprocessed by grayscale, OTSU binarization and other methods. Then the center of the microspheres was roughly located by Hough change method. It also has symmetry for the radial contours drawn from the exact center position in different directions, if it finds the phase of the same ring in different directions in the radial contours drawn from different center coordinate points, the coordinate of the point with the minimum variance of the phase is the center coordinate of the microsphere. Finally, combined with the sub-pixel theory, the measurement accuracy is further improved.

Firstly, the collected image is preprocessed, and the process and effect are shown in Figure 1. Firstly, a microsphere diffraction image is selected, and then a square image of 600*600 pixels is intercepted according to the position of the target. The threshold T obtained by OTSU method is 242, and the threshold segmentation is carried out according to the size of T to distinguish the image from the background.

The positioning effect is shown in Figure 2. Figure 2 (a) shows the result of directly processing the binarization image with Hough transform method, and the center point is (301, 297). In the figure, the pink center point is the center of the circle, and the green ring is the circle drawn with the radius obtained by detection. Then, the coordinates obtained by Hough transform method are restored to the original figure, figure 2 (b) shows the effect of positioning coordinates labeled to the original image.

Then, the radial contour curve of the microsphere image passing through the center of the circle was drawn, and the square image slightly larger than the spot target was cropped with the center of the image calculated by Hough transform method. The horizontal radial contour image passing through the center of the circle was taken, as shown in Figure 3. The red curve is the radial contour of the microsphere, and the blue dashed line corresponds to the first diffraction ring formed by diffraction. This part is the virtual

image generated by diffraction, and the light intensity is the strongest, and the influence of background noise is the least. In addition, the value of this part will not be offset, the fluctuation is obvious, and the noise interference is small, so it can be used as a fitting term.

Firstly, the position of this part of the curve corresponding to the blue dotted line is found, and then the phase corresponding to the peak is found by the method of quadratic equation fitting. The fitting method is to use the program to find the extreme point of the target position and take the same pixel distance coordinate points to the left and right sides of the extreme point as the center to fit. The size of the pixel distance should depend on the size of the microsphere, combined with the actual situation of the size of the microsphere used in the experiment, this paper studied the fitting range, and tested the fitting of different pixel ranges. The effect diagram is shown in Figure 4. The quadratic equation fitting of 7, 9 and 13 pixel ranges near the peak value is carried out respectively, and the fitting curve is compared with the actual data. It is found that the curve fitted by 9 pixel points has the smallest variance and is closest to the curve drawn by the original discrete points. Therefore, this study selects the peak as the center, 4 pixels on the left and right, and a total of 9 pixel ranges of data for fitting.

Then, the image was rotated every 30 degrees according to the center of the Hough transform, and the horizontal radial profile of the image was taken, that is, 12 radial profile curves were obtained from the same coordinate point, and the phase value corresponding to the curve peak of the first obvious diffraction ring of each curve was calculated, that is, the phase value of the 12 points where the red circle and the red dashed line intersect as shown in figure 5. In pixels, the variance of the phase is calculated.

According to the above method, the radial contour of eight coordinate points (300,296), (301,296), (302,296), (300,297), (302,297), (300,298), (301,298) (302,298) around the center point coordinate (301,297) is calculated respectively. The curve at the position of the first diffraction ring was fitted twice to obtain the phase value and calculate the variance. The results are shown in Table 1, where the phase variance of (300,298) coordinate point is the smallest, which is the exact integer pixel coordinate point.

Table 1. Phase variance of different center coordinates

Center point coordinates	Phase variance
300 296	0.003885
301 296	0.000986
302 296	0.001568
300 297	0.000836
301 297	0.001444
302 297	0.000755
300 298	0.000133
301 298	0.005042
302 298	0.004838

The high precision measurement of the integer pixel has been achieved by the radial contour symmetry method mentioned above, and then the linear interpolation method is used to enlarge the figure 10 times. The intensity value at each position is obtained by bilinear interpolation from four adjacent pixel values, so that the sub-pixel accuracy is 0.1pixel. Then, the radial contour phase variance method is repeated on the pixels around the center position, and the final coordinates are reduced by 10 times, which is the accurate sub-pixel center coordinates. The sub-pixel accuracy can be determined according to the number of surrounding pixels. The phase variance of 9 pixels with 0.1 pixel around the accurate center is selected, and the results are shown in Table 2, and the accurate center (299.9, 298.0) is obtained.

Table 2. Phase variance of different subpixel center coordinates

Center point coordinates	Phase variance
299.9 297.9	0.008423
300.0 297.9	0.000983
300.1 297.9	0.001763
299.9 298.0	0.000036
300.0 298.0	0.000456
300.1 298.0	0.000875
299.9 298.1	0.000143
300.0 298.1	0.004980
300.1 298.1	0.007293

In order to make the image closer to the actual situation, different background noises were added respectively to verify the effectiveness of the algorithm under different noises. The simulated images of speckle noise, salt and pepper noise and Gaussian noise were added respectively, to simulate the actual noise generated in the actual measurement environment, and then the positioning test was carried out according to the above method. The experimental results show that the measured horizontal XY coordinates are consistent with the actual coordinates, and can achieve sub-pixel accuracy, which proves the accuracy of the algorithm.

Z direction position measurement

Michael et al. proposed a defocus imaging method based on experimental images in 2003. The experimental images were collected by agarose gel with a diameter of 216nm. The least square method was used to fit the relationship between the outermost ring radius r0 and the defocus distance r of the microspheres. The corresponding axial position can be obtained by finding the outermost ring radius R of the measured microsphere, and the axial resolution of 5m can be measured by this method at that time. When the fluorescent particles are used for measurement, the bright particles are measured in the dark environment. There is a significant gap between the light intensity of the particles and the background, so it is very easy to distinguish the microsphere image from the background. So the simple edge detection and image segmentation methods can be used to obtain the outermost ring radius. It is difficult to extract the location of the outermost ring radius. Based on this, the relationship between the radius of the inner ring of the microsphere spot and the defocus distance is studied, and an improved method is proposed.

First, find the spot radius of the microsphere, find the relationship between the spot radius and the defocus distance, and find the location of the first obvious diffraction ring, which is the inner ring radius of the spot. Due to the CCD imaging of dark microspheres in liquid environment under bright background, the background noise cannot be eliminated by optical design, so the radial profile is not only the light intensity of the diffraction spot of the microspheres, but also the light intensity of the background noise. Therefore, the radial profile is averaged according to different angles, and then the obtained radial profile curve is fitted. The influence of background noise can be eliminated. After obtaining the radius of the target light spot of each microsphere, it was arranged according to different defocus distances. In this study, 100 images were selected, and the distance between each image was driven by the nano displacement table, which was 50nm. The scatter plot was drawn, as shown in Figure 7, red scatter.

The results show that not only the radius of the outermost ring is proportional to the axial defocus distance z, but also the other coaxial rings are proportional to the defocus distance. The diffraction virtual image with the most stable light intensity is selected to create the model, and the linear relationship is . After the measured image is selected to obtain accurate lateral coordinates, the radial contour is drawn, and the target position is fitted to obtain the radius size, and the corresponding axial position can be obtained according to the linear relationship.

After obtaining the three-dimensional coordinates of the microspheres according to the above algorithm, the position coordinates of the microspheres in the continuous images were connected together in a time

series to obtain the motion trajectory as shown in figure 6.

Three-dimensional step test

In order to check the true resolution of the proposed method in X, Y and Z directions, a 10\mu m size microsphere was step tested in three directions, respectively. The microspheres were placed on the bottom of the slide and placed on a 3D piezoelectric displacement stage, and three stages of continuous motion were recorded by a CCD camera in three different directions using the software included in the displacement stage, thus achieving 3D positioning of the microspheres. Because driving in all three directions at the same time creates coupling, experiments in each direction are required separately. In the X, Y, and Z directions, the displacement stage was repeatedly round-trip at a frequency of 1Hz with a step size of 50nm, and it was tracked using a high-pass filter to obtain a step change of 50nm in the XY and Z directions, as shown in figure 7. Ideally, the resolution of the 3D position of the measured microspheres should be close to the simulated value and have sub nanometer resolution. However, in practical applications, experiments will produce coupling phenomena, which will inevitably generate ambient noise, which will affect the accuracy of the measurement. In the experiment, although the displacement table is stationary, there will be a relative movement between the objective lens and the sphere that can be calculated, and this movement amplitude is about tens of nanometers per minute. This is mainly because the position of the objective lens and the displacement table are offset, which leads to systematic errors. The actual measurement accuracy is higher than the detected value.

Cardiomyocyte binding experiment

The microsphere is combined with the cardiac muscle cell, and the three-dimensional nano displacement table is driven by a step of 50nm. The image collected on the defocus plane is shown in Figure 8, where the black and white aperture is the diffraction spot of the microsphere, and the background is the cardiac muscle segment. It is difficult to directly observe the motion state of the microsphere and the cell through the microscope, and through the standard image processing technology, The center region of each microsphere is segmented, and the three-dimensional position measurement is carried out. Then the positions of adjacent frames are connected to obtain the three-dimensional motion information of the microsphere, which indirectly reflects the cell dynamics. The minimum motion distance we collected is 50nm, and when the motion distance is 50nm, the lateral coordinate produces 1 pixel change, and when the motion distance is 100nm, the axial coordinate produces 1 pixel change. Experimental results show that with our current experimental system and computational algorithm, we can achieve a resolution of 50nm in the lateral direction and 100nm in the axial direction. In order to further eliminate the interference of the substrate on the diffraction points, by improving the stability of the displacement mobile platform and improving the sampling speed and efficiency, we believe that the resolution will be further improved by an order of magnitude or even higher. Under the conditions of high disturbance and bright field in liquid state, the three-dimensional positioning of dark particles can reach the accuracy of 50nm in the horizontal direction and 100nm in the vertical direction, which will fill the gap in the measurement direction of non-damaging nano-scale accuracy tracking in live cells. In the future, there can be more

extensive research in live cell detection and cell drug therapy. Compared with the usual use of fluorescent particles for three-dimensional positioning research can be reduced experimental conditions. Microspheres and their substrates are no longer limited to transparent materials, and future high-precision small-scale measurements of microspheres will be applied to a wider range of scenarios, not only in the field of biological detection, but also in actual industrial production and measurement.

Summary and outlook

Due to the continuous development and progress of single event tracking technology, a variety of three-dimensional positioning and tracking measurement methods in different application fields and types have also emerged. However, for the study of living cells, there is a higher demand for the development of a non-damaging technique using computer vision analysis, not limited by spatial resolution or adverse perturbations to tissues. To address the technical limitations of current measurements. In order to realize the fast and accurate three-dimensional position measurement of dark microspheres in the bright field of liquid environment where cardiomyocytes exist, this paper makes up for the shortcomings that the existing research methods of three-dimensional positioning of microspheres can only measure transparent samples and transparent substrates, and the measurement range and application scene are greatly limited. An improved lossless detection method is proposed in this paper. This mark-free detection method can obtain three-dimensional position measurement with an accuracy of 50nm in XY direction and 100nm in Z direction. There is no restriction on the material of the microsphere itself, and the substrate no longer needs to be the same material as the microsphere. The measurement range of the microspheres will be greatly increased.

Declarations

Acknowledgements This work was supported by the National Natural Science Foundation of Liaoning (Grant Nos. 2020-MS-219).

Compliance with ethical standards

Conflict of interest on behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Neutsch K, Schnitzler L, Sun J, Tranelis MJ, Hofmann MR, Gerhardt NC. In-depth particle localization with common-path digital holographic microscopy. In: Practical Holography XXXIV: Displays, Materials, and Applications. Vol 11306. 2020;53-58. doi:10.1117/12.2545925
2. Nahorniak M, Pasetto P, Greneche JM. Two-step single-reactor synthesis of oleic acid- or undecylenic acid-stabilized magnetic nanoparticles by thermal decomposition. Beilstein J Nanotechnol. 2023;14:11-22. doi:10.3762/bjnano.14.2

3. Jaesung P, Sunghoon B, Taeho J, Cheolwoo P, Ho L. In vivo cell tracking using speckle image correlation technique employing high frame rate confocal laser scanning microscopy in a mouse skin model. J Mech Sci Technol. 2019;33(6):3039-3050. doi:10.1007/s12206-019-0554-0
4. Wu S, Li Y, Ding W, Xu L, Ma Y, Zhang L. Recent Advances of Persistent Luminescence Nanoparticles in Bioapplications. Nano-Micro Lett. 2020;12(1):70. doi:10.1007/s40820-020-0404-8
5. Scitovski R, Sabo K, Nikić P, Majstorović S. A new efficient method for solving the multiple ellipse detection problem. Expert Systems with Applications. 2023;222:119853. doi:10.1016/j.eswa.2023.119853
6. He W, Wu G, Fan F, Liu Z, Zhou S. A Robust Real-Time Ellipse Detection Method for Robot Applications. Drones. 2023;7(3):209. doi:10.3390/drones7030209
7. Ou Y, Deng H, Liu Y, Zhang Z, Lan X. An Anti-Noise Fast Circle Detection Method Using Five-Quadrant Segmentation. Sensors. 2023;23(5):2732. doi:10.3390/s23052732
8. Leister R, Fuchs T, Mattern P, Kriegseis J. Flow-structure identification in a radially grooved open wet clutch by means of defocusing particle tracking velocimetry. Exp Fluids. 2021;62(2):29. doi:10.1007/s00348-020-03116-0
9. Liu Y, Deng H, Zhang Z, Xu Q. A Fast Circle Detector with Efficient Arc Extraction. Symmetry. 2022;14(4):734. doi:10.3390/sym14040734
10. Jianing W,Jianing C.Subpixel edge detection algorithm based on improved Gaussian fitting and Canny operator. AJCIS. 2022;5(7). doi:10.25236/AJCIS.2022.050706
11. Dou W, Zhao Q, Malhi M. Label-free conduction velocity mapping and gap junction assessment of functional iPSC-Cardiomyocyte monolayers. Biosensors and Bioelectronics. 2020;167:112468. doi:10.1016/j.bios.2020.112468

Figures

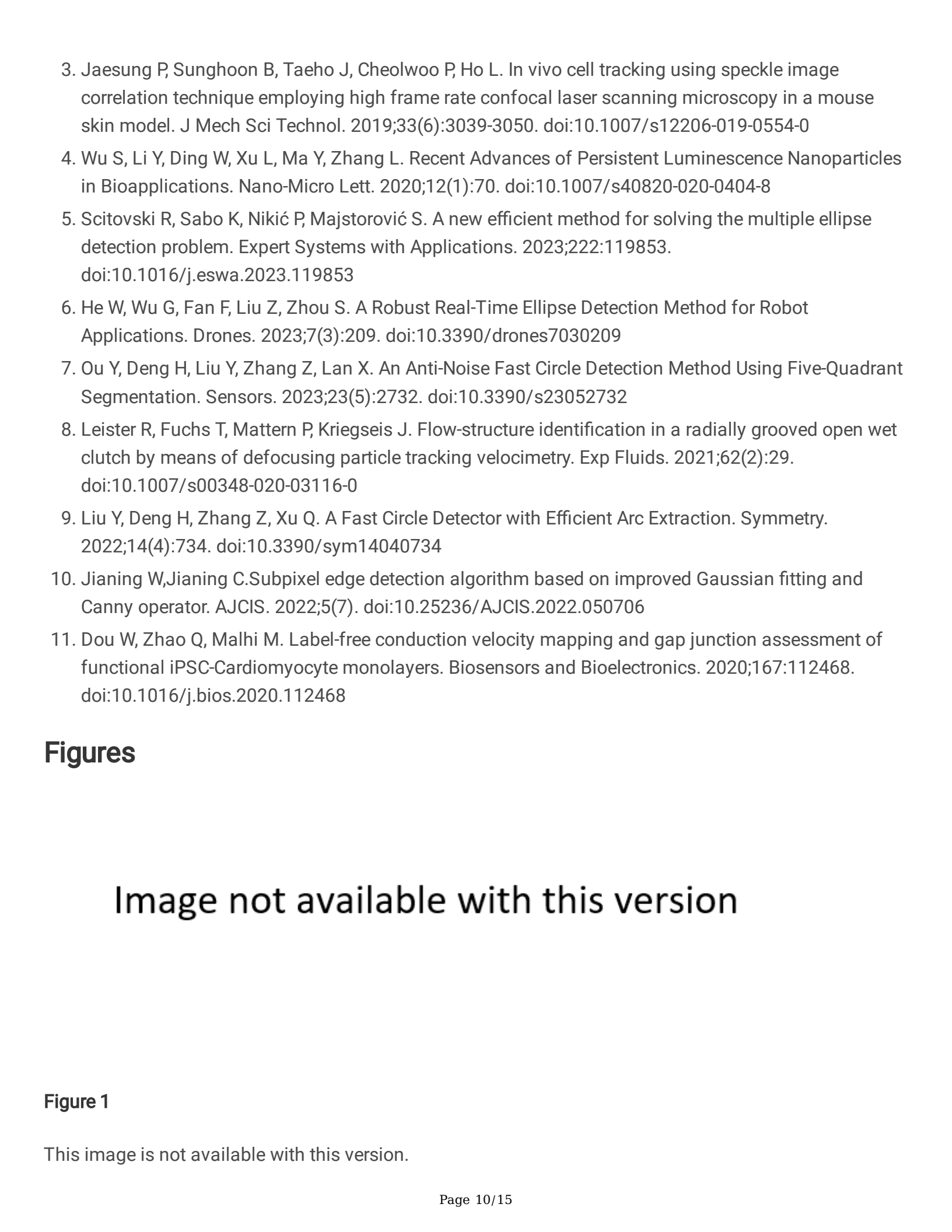

Figure 1

This image is not available with this version.

Image not available with this version

Figure 2

This image is not available with this version.

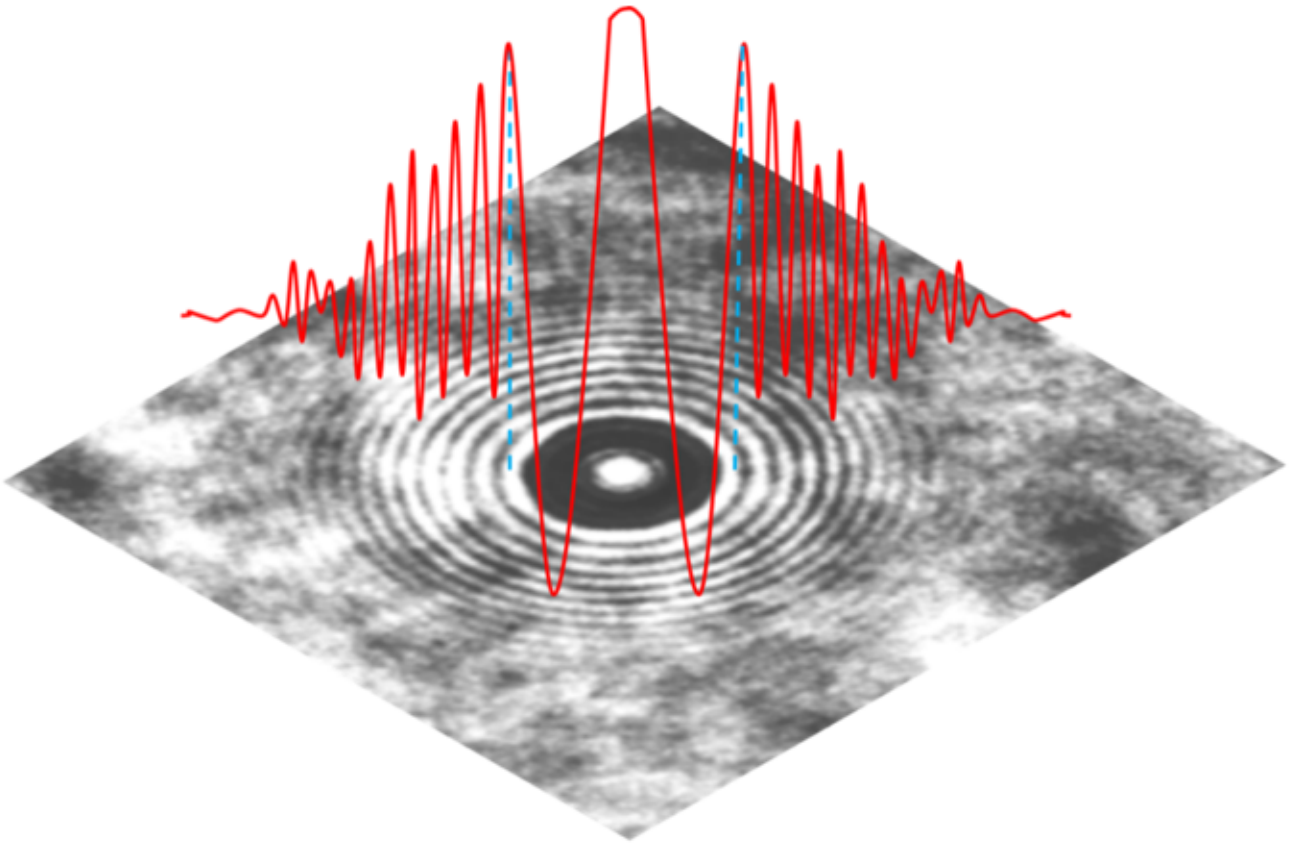

Figure 3

Fitting diagram of spot radius of microsphere ring.

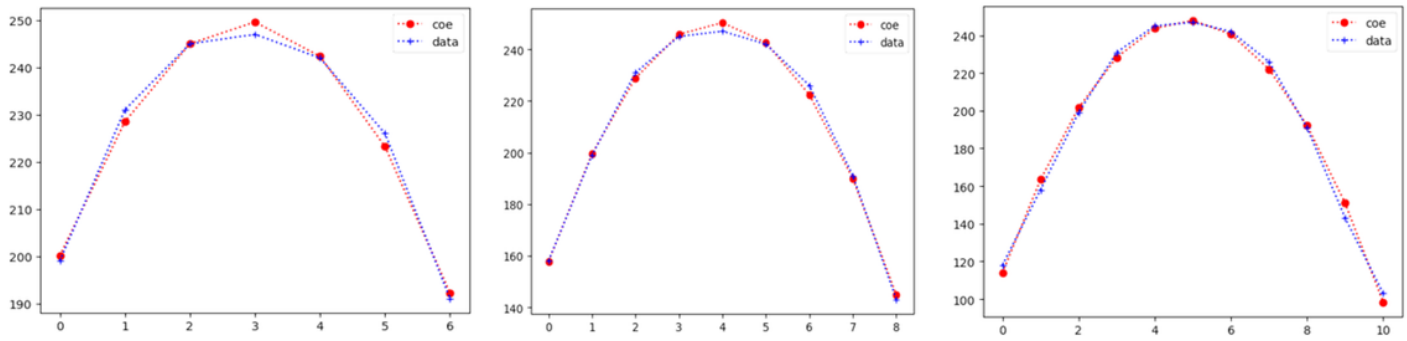

Figure 4

Fitting diagram of light intensity curves in different ranges.

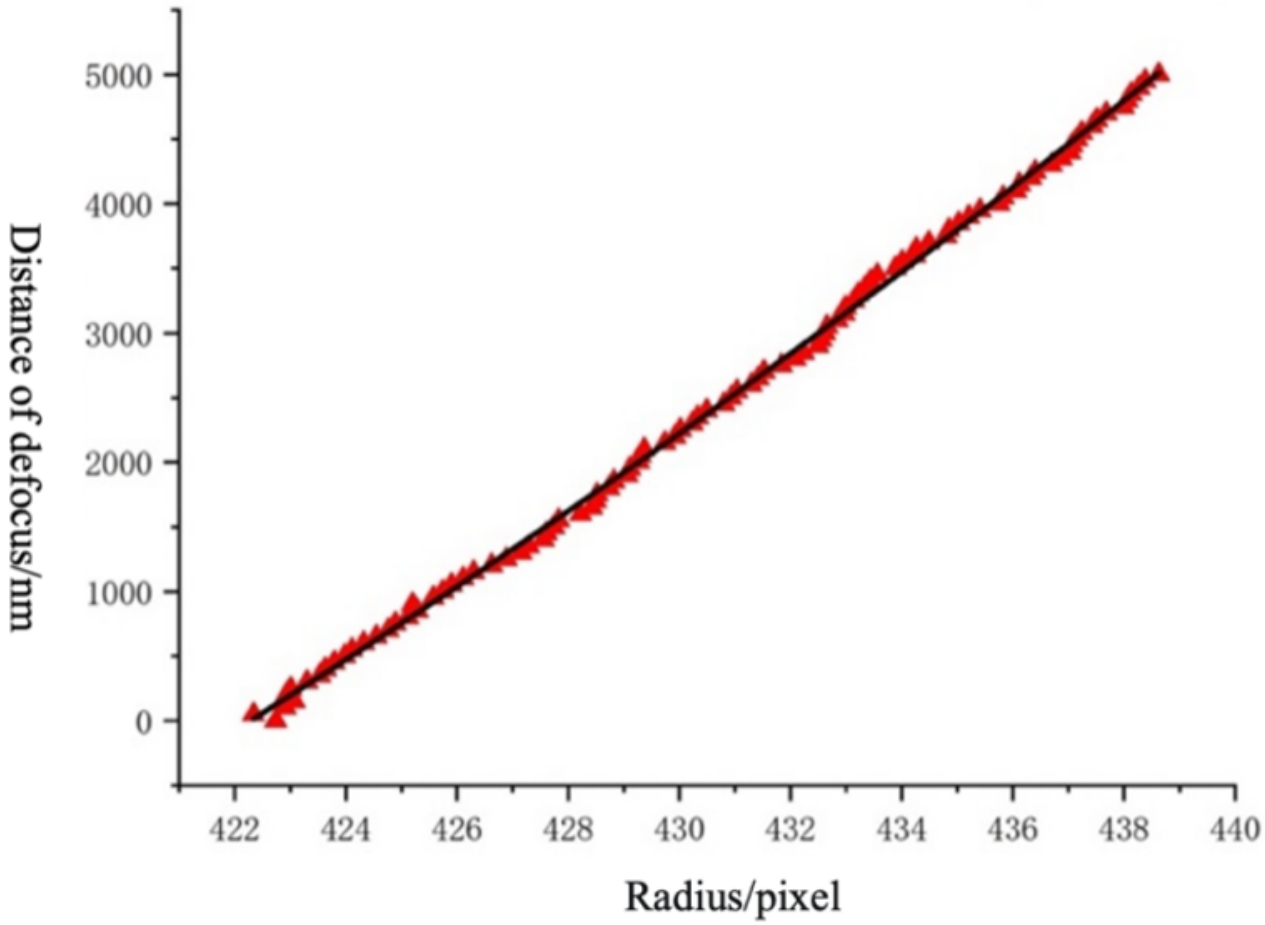

Figure 5

Relation between inner ring radius and defocusing distance of microsphere.

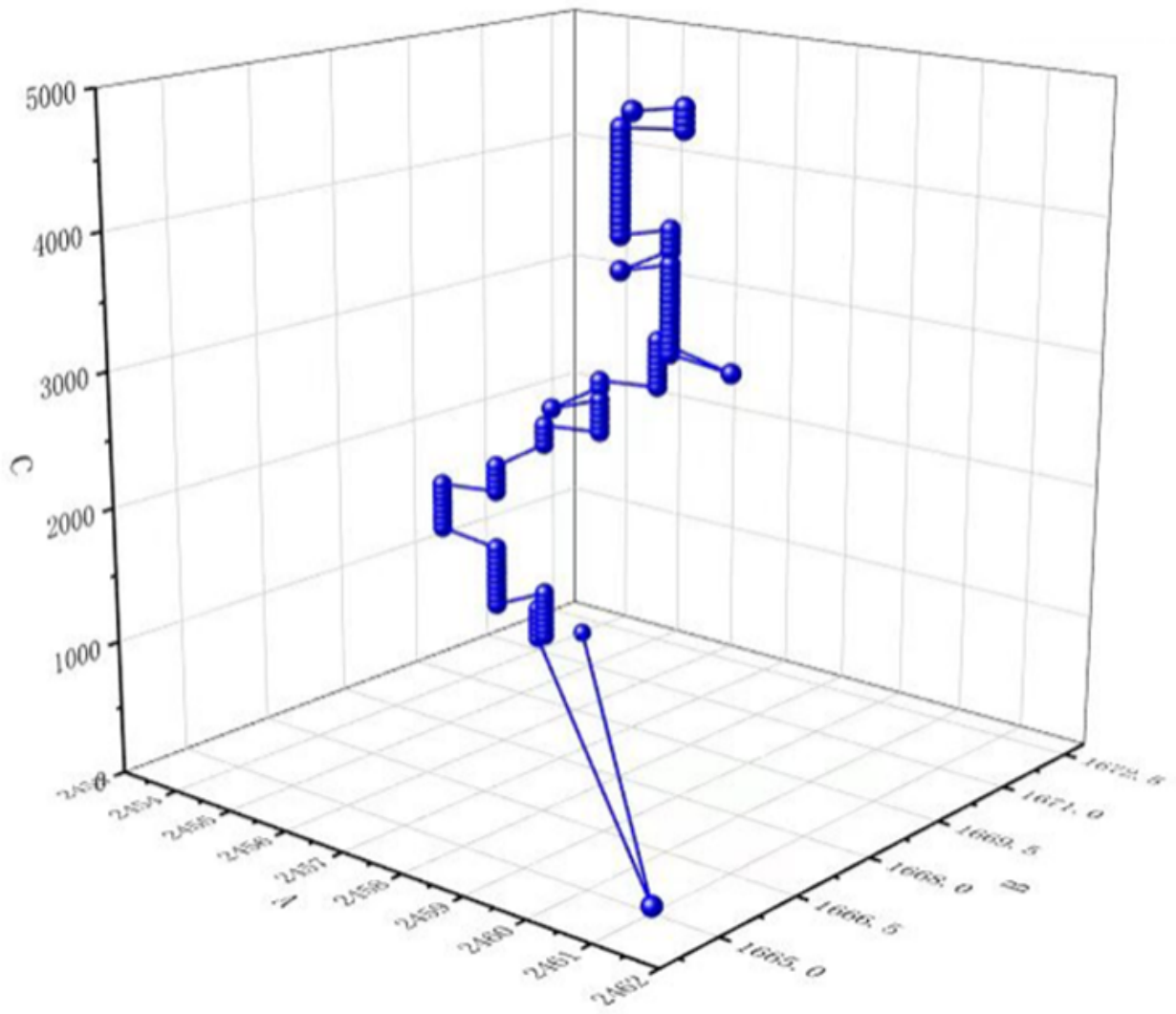

Figure 6

Three-dimensional motion trajectory of the microsphere.

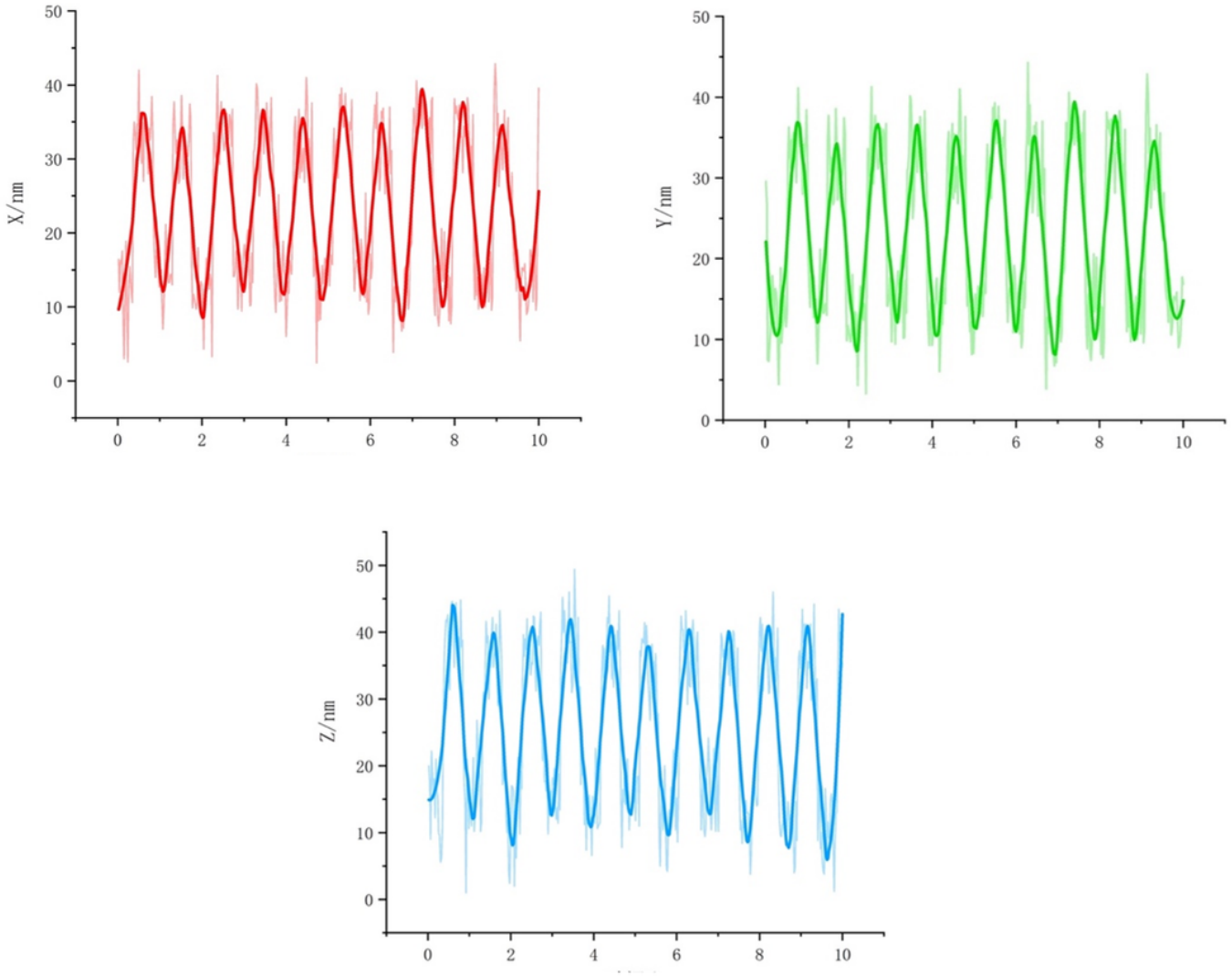

Figure 7

XYZ nanometer step measurement.

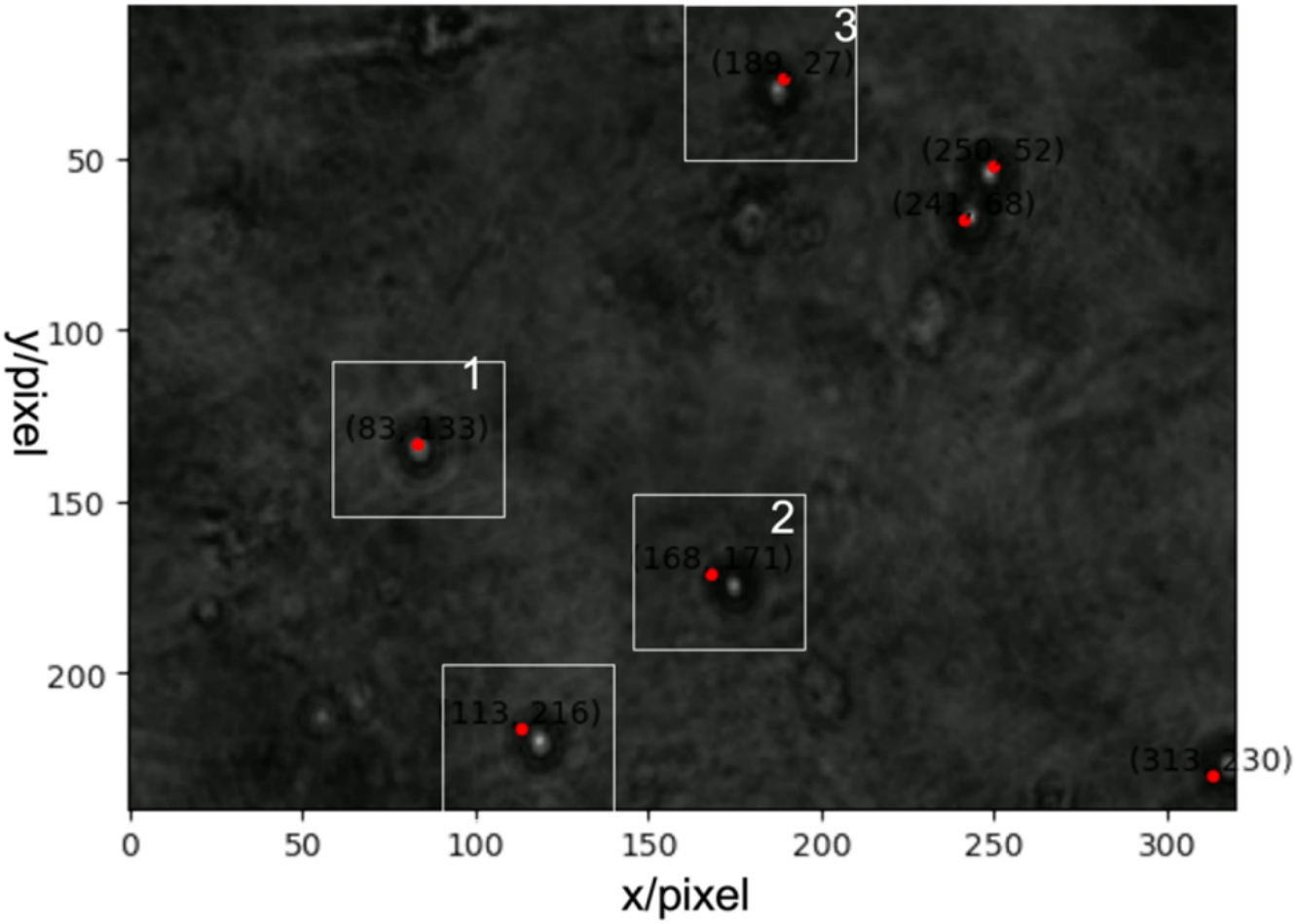

Figure 8

Image of myocardial cells combined with microspheres.