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# Enhanced 3D localization of individual RNA transcripts via astigmatic imaging

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## ABSTRACT

Here we present an automated microscope capable of 3D multi-color single molecule localization of individual messenger RNA molecules in a wide range of cell types. We have implemented astigmatic imaging with a cylindrical lens to improve  $z$ -localization, and a maximum likelihood estimator on a graphics processing unit to improve localization precision and speed. This microscope will aid in gene expression analysis by its capability to perform high throughput imaging of thick cells and tissues while still maintaining sufficient  $z$  resolution to resolve single RNA transcripts in three dimensions. Enhanced  $z$ -localization allows for resolving membrane localized and co-localized transcripts.

**Keywords:** smFISH, single-molecule localization, astigmatic imaging, fluorescence microscopy, gene expression

## 1. INTRODUCTION

Measurement of signal transduction networks by fluorescence microscopy has enabled quantitative measurements of key signaling molecules (such as messenger RNA, proteins, etc...) and prediction of the expression of these molecules in response to varying external stimuli and/or biological modifications. One particularly powerful example is the measurement of genetic regulatory networks through fluorescent labeling and counting of single RNA transcripts.<sup>1,2</sup> Applying quantitative imaging and system dynamics techniques to biological systems have provided a new understanding of chromatin remodeling,<sup>3</sup> spatial patterning of gene expression during development,<sup>4-6</sup> prediction of signal-activated gene expression,<sup>7</sup> the role of long non-coding RNA,<sup>8,9</sup> digital expression of small RNA in pathogenic bacteria.<sup>10</sup> As this experimental/computational combination further pushes the boundary of our biological knowledge, the ability to acquire multi-dimensional high fidelity data becomes increasingly important.

A variety of fluorescent labeling techniques are currently in use to tag individual bio-molecules for tracking and quantification.<sup>1,2,11-16</sup> Digital counting of individual messenger RNA (mRNA), is commonly accomplished using a variant of fluorescence in-situ hybridization (FISH<sup>1,2,10,17</sup>). In particular, single-molecule FISH (smFISH<sup>2</sup>) has gained popularity due to the simplicity of probe design and wide variety of fluorescent dyes. Recent advancements in probeset design now allow for the detection of single-nucleotide polymorphisms (SNPs<sup>18,19</sup>), short (<200 nucleotide) RNA targets,<sup>10</sup> highly multiplexed measurements,<sup>17</sup> and alternative splicing.<sup>20</sup> smFISH experiments utilize probesets consisting of 20-30 short oligos (20-22 nucleotides each), individually conjugated to a fluorescent dye (e.g. Cy3, Cy5, Alexa 488). Two drawbacks of the smFISH technique are 1) it is necessary to fix and permeabilize cells prior to labeling, requiring a number of independent experiments to obtain temporal dynamics and 2) the data is primarily two dimensional, due to the lack of axial resolution typical of wide-field imaging. We have previously addressed the first drawback by creating an automated, multi-color, single-molecule localization microscope package<sup>10,21</sup> and others have shown that measuring gene expression at key time points is more

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valuable in discerning system dynamics<sup>22</sup>. We note that overcoming the first limitation by creating an automated, multi-color imaging system has created a third drawback: extremely large and computationally costly datasets, requiring orders of magnitude more time to process than generate.

In this paper, we address both the axial and computational drawbacks outlined above by altering our microscope to perform astigmatic imaging<sup>23</sup> and altering our data analysis package to extract three-dimensional localizations through graphics processing unit (GPU) computing. The former technique provides an approximate 200 nm accuracy in the axial direction and the latter speeds analysis of our large datasets by roughly four orders of magnitude. We demonstrate the improved axial resolution (from our original epi-fluorescence microscope) and data analysis through a calibration sample of fluorescent microspheres and then apply this technique to detect gene expression of key transcription factors in mesenchymal stem cells differentiating into bone tissue.

## 2. EXPERIMENTS

### 2.1 Microsphere calibration

To calibrate the  $z$  position locating capability of our astigmatic imaging system we used 20 nm fluorescent beads (Invitrogen Fluospheres F-878) adhered to a coverslip in a monolayer by a spin coating process. Using a Physik Instrumente piezo objective scanner (PI P721.20) successive images were taken at different planes of focus with increments of 50 nm. For each image at a given plane of focus the point-spread function (PSF) from individual beads was processed in Matlab using a 2D Elliptical Gaussian Maximum Likelihood Estimator (MLE).<sup>24</sup> 18 by 18 pixel (or 1.3  $\mu\text{m}$ ) windows were selected from the original image centered over single beads and processed using the MLE algorithm to extract elliptical parameters,  $w_x$  and  $w_y$ . A total of 40 beads selected over a 36  $\mu\text{m}$  by 36  $\mu\text{m}$  area were fit at each plane in the image stack and averaged to yield  $w_x$  and  $w_y$  as a function of focal plane position. It was assumed that across the 36  $\mu\text{m}$  area the bead  $z$  position does not vary significantly. The discrete measured data was fit to a defocus curve using a least squares optimization routine native to Matlab. The fitting form and procedure was adapted from previous work.<sup>23,25</sup>

### 2.2 smFISH probesets

Probe sets for RunX2 were purchased from Biosearch Technologies, utilizing a probe designer that maximizes G-C content of individual probes while ensuring minimal nonspecific binding. For all probes Cal Fluor 590 dye was used as the fluorescent reporter. Probe sets were diluted to 100  $\mu\text{M}$  concentrations in nuclease-free water and aliquoted into 25  $\mu\text{L}$  batches for subsequent experiments. The sequences of all probes are available upon request.

Hybridization of the probes to target mRNA was performed in a hybridization buffer that consisted of 1 g dextran sulfate, 10 mg *Escherichia coli* tRNA, 100  $\mu\text{L}$  of 200 mM vandial ribonucleoside complex (NEB), 40  $\mu\text{L}$  of 50 mg/mL bovine serum albumin (BSA) (RNase free) (Ambion), 1 mL 20x saline-sodium citrate (SSC) (RNase free, Ambion), 3 mL formamide (deionized, Ambion), with a final solution volume of 10 mL. We arrived at the 30% formamide concentration by performing a dilution series of 10-40% formamide in 10% increments, with 30% yielding the best signal-to-noise ratio. Sample washing was performed in a buffer consisting of 5 mL 20x SSC (RNase free, Ambion), 15 mL formamide (deionized, Ambion) and 20 mL nuclease-free water.

### 2.3 Mesenchymal cell culture and fixation

Adipose-derived human mesenchymal stem cells (ATCC) were cultured on 1  $\text{cm}^2$  borosilicate chamber slides (Nunc) in mesenchymal cell culture basal medium (ATCC) supplemented with 10% fetal calf serum (Hyclone) and Glutamax (Invitrogen). Cells were seeded and differentiated into osteocytes according to manufacturers directions (StemPro Osteo Differentiation Kit, Invitrogen). Cells were differentiated for 0, 7, 14, and 28 days, fixed with 4% paraformaldehyde, and then incubated overnight in ethanol at 4  $^{\circ}\text{C}$  prior to labeling.

## 2.4 smFISH hybridization

The ethanol was aspirated off and 400 mL of the wash buffer was added to each chamber. After 5 minutes, the wash buffer was aspirated and 400 mL of new wash buffer added. The probe set was then diluted in hybridization buffer to a dilution of 1:1000. 300-400 mL of probe solution was added to each chamber and incubated overnight at 30 °C. In the morning, the hybridization buffer was aspirated, 400 mL of the wash buffer added to each chamber, and incubated for 30 minutes at 30 °C. The wash buffer was aspirated, 400 mL of wash buffer containing 5 ng/mL of DAPI was added to each chamber, and incubated for 30 minutes at 30 °C. The final wash buffer was aspirated and 400 mL of 2x SSC was added to each chamber.

## 2.5 Multiplexed astigmatic imaging

Multiplexed imaging was performed on an Olympus IX71 inverted platform using a mercury arc lamp (Olympus U-LH100HGAPO) for excitation over the spectral range of 400-900nm. An automatic 6 position dichroic filter and controller (Olympus IX2-RFACA) was used to programmatically switch between filter sets used for the experiment. The filters used were DAPI (DAPI-1160B-000), Cy3 (CY3-4040C-000), and Cy5 (CY5-4040C-000). An Olympus PlanFluor 100X 1.3NA objective was used for imaging, mounted atop a high resolution piezo scanner for  $z$ -position control (PI P721.20). The sample held in a LabTek 8-well chamber plate was secured to a custom adapter mounted on an  $(x, y)$  positioning stage (Thorlabs MAX200). To form an astigmatic pointspread function (PSF) a low power cylinder lens with 1000mm focal length (Thorlabs LJ1516RM-A) was placed 50mm before the camera. Images were captured with a Scientific CMOS camera (Hamamatsu C11440-22CU) with a sampling of 2048 by 2048 pixels. To switch between astigmatic and normal imaging the cylindrical lens was mounted in a motorized flipper stage (Thorlabs MFF101).

All of the hardware was interfaced with LabVIEW for data acquisition. Custom LabVIEW software allowed for fully automated collection of variable  $z$ -stacks for each of the 6 possible filter positions with unique acquisition parameters selectable for each filter. The camera and  $(x, y)$  stage were connected through USB while the  $z$ -piezo and flipper motor were connected to a general purpose DAQ (NI PCIe-6343) for analog and digital outputs respectively. All hardware was software timed and runs in a completely automated manner until the experiment is completed. The software operates in a nested loop structure, stepping through the four experimental inputs,  $x$  position,  $y$  position, filter setting, and  $z$  position. For the experiments described we used an  $(x, y)$  grid of 3 by 3 points with 300  $\mu\text{m}$  increments and three filters, DAPI for nucleus, Cy3 for the FISH signal, and Cy5 for a membrane stain.  $Z$ -stack sizes varied depending on the imaging mode, with 40 images per stack with 500 nm steps for normal imaging, while astigmatic imaging was 20 images per stack with 1  $\mu\text{m}$  steps.

Data analysis was performed using a modified version of custom software written in Matlab by our group.<sup>10,21</sup> To accommodate astigmatic imaging we use a modified version of the code which calculates a more accurate  $z$  position for every located FISH spot as opposed to assigning its location to the  $z$  plane with the highest intensity. We fit every possible fluorescent spot simultaneously using a graphics processing unit (Nvidia GTX 760), utilizing modified code from the Lidke group.<sup>24</sup> This provides all parameters necessary to calculate the  $z$ -position,  $x_0, y_0, w_x, w_y$ , respectively the  $x$  and  $y$  centroid and  $x$  and  $y$  width of the elliptical gaussian fit by the GPU. Calculation of the  $z$  position using the astigmatic PSF shape is explained in the results section 3.1. The additional computational time added from  $z$  position finding is less than a minute for a typical experiment with 10,000-30,000 FISH spots. Astigmatic FISH spots are assigned to cellular compartments by comparing the localized FISH spot position  $(x, y, z)$  to the nuclear and membrane masks generated from DAPI and Cy5 stains respectively. Co-localized spots are found by thresholding intensities 1.5 times greater than FISH spot intensities. Spots that are too large or below the FISH threshold are not further processed and do not get assigned to cellular compartments.

## 3. RESULTS

### 3.1 Axial resolution of astigmatic imaging system

Without astigmatic modifications, the axial resolution of our microscope<sup>10,21</sup> is approximately 1.2  $\mu\text{m}$ , limited primarily by out-of-focus fluorescence (Fig. 1A). This restricts our ability to co-localize RNA spots, determine membrane bound RNA, and localize RNA spots in high-background and/or thick cell samples. Motivated by the

advancements in 3D single molecule localization by astigmatic imaging,<sup>23,25</sup> we introduce a weakly defocusing cylindrical lens into the emission pathway (Fig. 1B) to introduce an astigmatism to our point-spread function (PSF). Figure 1C shows the result of this modification for multiple focal planes above and below a fluorescent microsphere on No. 1 cover glass. There is clearly a correlation between the ellipticity and position of the microsphere in relation to the focal plane. To utilize this elliptical PSF, it is required to 1) fit the PSF in each focal plane with an elliptical gaussian, 2) calibrate the ellipticity to the known distance from the focal plane, and 3) create a calibration curve by performing (1) and (2) for many individual microspheres.

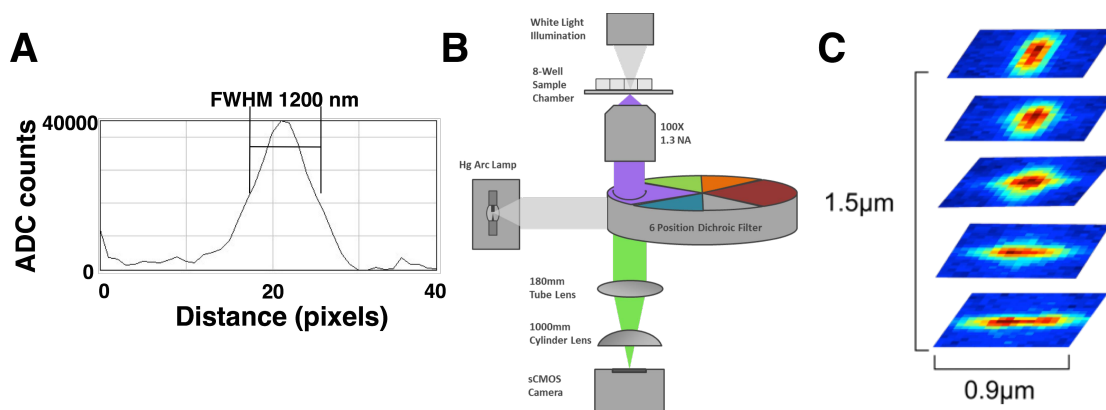


Figure 1. Results of adding a weakly defocusing cylindrical lens. A) Axial PSF without astigmatic lens. B) Modified optical path in astigmatic microscope. C) Focal imagestack of a single fluorescent microsphere with astigmatic imaging system.

To determine the focal plane offset of any given elliptical PSF, we use custom Matlab code based on work by the Lidke and Bersdordf groups<sup>24,26</sup> that performs a maximum-likelihood fit (on the GPU) of a 18x18 matrix surrounding each spot to,

$$G(x, y) = H \exp \left[ -\frac{2(x - x_0)^2}{w_x^2} - \frac{2(y - y_0)^2}{w_y^2} \right] \quad (1)$$

then use the average of all fits at each focal plane to fit a 5<sup>th</sup> order defocus curve,

$$w(z)_{x,y} = w_{0,x,y} + A(z - c/d)^2 + B(z - c/d)^3 + C(z - c/d)^4 + D(z - c/d)^5 \quad (2)$$

and finally determine the focal plane offset by minimizing,

$$D(z) = (w_x^{1/2} - w(z)_{x,calibration}^{1/2})^2 - (w_y^{1/2} - w(z)_{y,calibration}^{1/2})^2 \quad (3)$$

using the function *fminsearch* in Matlab. Here  $x$  and  $y$  are individual pixels in the 9x9 matrix,  $x_0$  and  $y_0$  the center coordinates of the elliptical PSF,  $w_x$  and  $w_y$  the width of the PSF in each dimension, and  $H$  the intensity. Because we perform these operations on the GPU, we are able to fit every potential fluorescent spot in the image at once, drastically speeding both localization and spot identification. For roughly 30,000-100,000 potential smFISH targets, we are able to perform all MLE fits on the order of minutes.

Figure 2 shows the results of applying Eqs. (1)-(3) to fluorescent microspheres. Figure 2 shows averaged  $G(x, y)$  for  $w_x$  (blue circles) and  $w_y$  (red circles) and the corresponding fit of  $w(z)_x$  (blue line)  $w(z)_y$  (red line). Equations (1)-(3) are sufficient to localize individual fluorescent emitters in  $(x, y, z)$  below the diffraction limit. An extensive review of localizing fluorescent emitters below diffraction limit in  $(x, y)$  using MLE can be found ref 27 and here we will focus on our results in the axial ( $z$ ) dimension. Measuring a new sample of fluorescent microspheres (Fig. 3), with a different size and wavelength than our original sample, we find a localization accuracy of 200 nm within  $\pm 1 \mu\text{m}$  of the focal plane. Samples with individual fluorophores on multiple focal planes, such as thick biological samples, introduce extra complexity in the data analysis. Fluorophores may be at similar  $(x, y)$  positions, yet be separated in the axial dimension. Due the large FWHM of our non-astigmatic

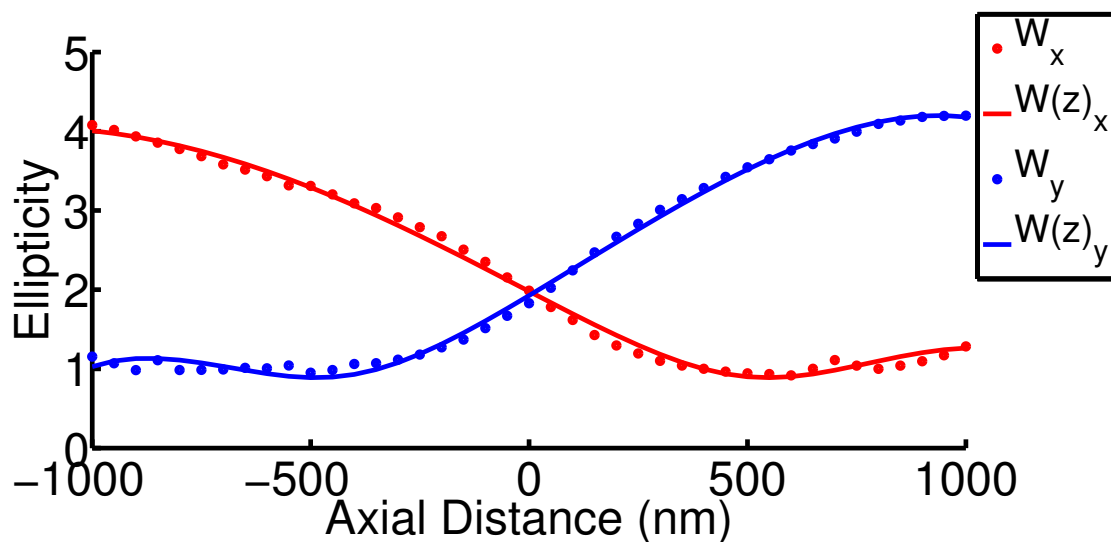


Figure 2. Calibration of astigmatic imaging using fluorescent microspheres.  $w_x$  (blue circles) and  $w_y$  (red circles) cross-over at the focal plane and Eq (2) fits the observed values of  $w_x$  (blue line) and  $w_y$  (red line) well over a  $2\ \mu\text{m}$  range.

microscope ( $\sim 1.2\ \mu\text{m}$ ), it is almost impossible to separate individual fluorophores in the axial dimension. Given the increased axial resolution of our astigmatic microscope ( $\sim 200\ \text{nm}$ ), we are able to determine the axial position of individual fluorophores. To accomplish this task, we take fewer images in each focal stack (20 vs. 40), individually threshold each focal plane instead of a maximum intensity projection, and finally assign fluorophores to the focal plane where the parameters obtained from the MLE are closest to those obtain for an in-focus fluorophore (middle image in Fig 1C).

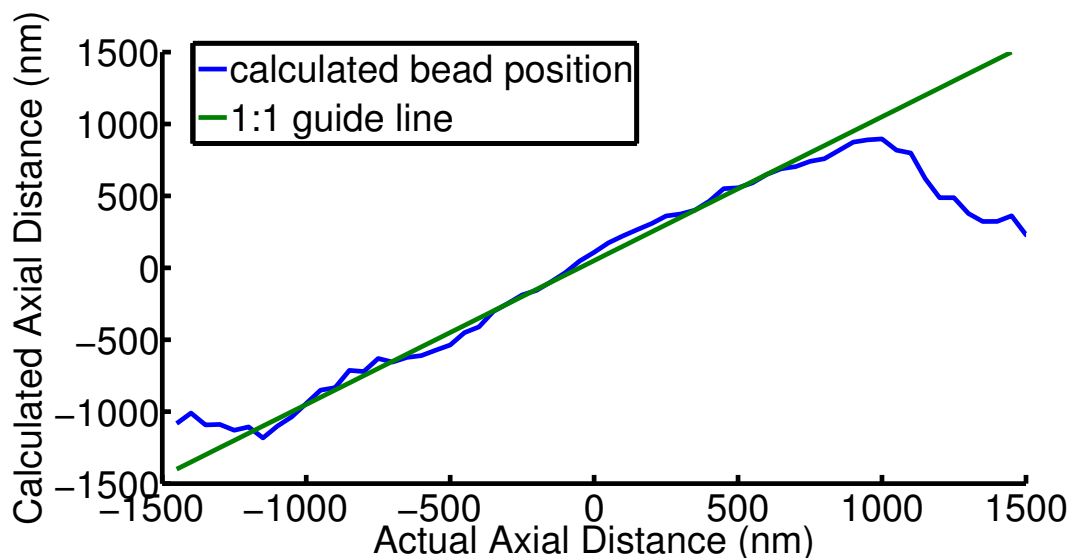


Figure 3. Z localization of fluorescent microspheres and error in localization. We achieve good axial localization of our calibration sample within the  $2\ \mu\text{m}$  usable range, with a maximum error of  $200\ \text{nm}$  and an average error of  $75\ \text{nm}$ .

### 3.2 Three-dimensional gene expression in MSCs

The nearly 6-fold improvement in axial localization precision afforded by astigmatic imaging lends itself naturally to 3D localization of individual mRNA in thick biological cells or tissues. In particular, while many groups have shown the importance of spatial patterning of RNA in two dimensions,<sup>4-6</sup> the extension to three dimensions remains limited. Improved axial resolution is critical for determining transcriptional regulation in confluent cells that display emergent behavior. Here we will focus on mesenchymal stem cells (MSCs) differentiating to bone,<sup>28</sup> as process known as osteodifferentiation. During osteodifferentiation, MSCs under-go a wealth of transcriptome changes that are indicative of bone creation.<sup>29,30</sup> Ensemble measurements (such as PCR) have shown differential expression in multiple genes, such as RunX2,<sup>30-34</sup> during osteodifferentiation. However, while the identity of key genes responsible for osteodifferentiation have been identified, this information is insufficient to predict bone yield and/or suggest possible chemical, surface, or biological stimulants that promote osteodifferentiation. This is most likely due to the highly heterogeneous spatial and temporal response of individual MSCs. A key step to understanding how different stimuli affect bone yield is to measure the distribution of single-cell genotype and phenotype as osteodifferentiation proceeds.

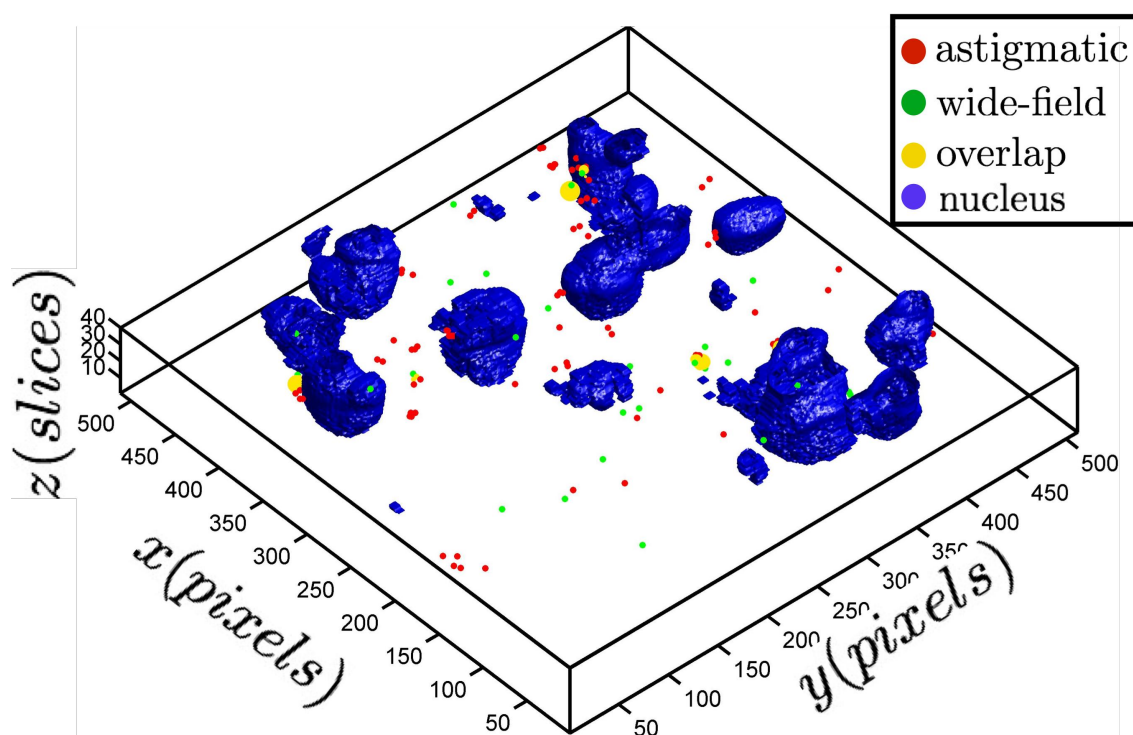


Figure 4. 3D voxel representation of the same area of 14-day growth, confluent MSCs with both wide-field and astigmatic imaging techniques. The  $x$  and  $y$  dimensions are in pixels and the  $z$  dimension is given in focal planes or "slices". RunX2 mRNA are represented as red voxels for astigmatic imaging, green voxels for wide-field imaging, and the nuclei are blue voxels. Spots that overlap in  $(x, y)$  are shown in yellow. For the overlap the sphere size is directly proportional to  $z$ -position difference (ranges from 0 to 5 focal planes). The average  $z$ -position difference between astigmatic and normal was 800 nm and 50% of the overlapping spots had a  $z$  difference close to 250 nm.

Here we count and localize in three dimensions RunX2 mRNA, a key transcription factor that has previously been shown to have a temporal and spatial dependence during the osteodifferentiation process.<sup>29</sup> To promote osteodifferentiation, we stimulate a population of confluent MSCs at day 0 (see section 2.3) and image the cells every seven days. To test our hypothesis that astigmatic imaging will allow for detection of fluorescent-tagged mRNA transcripts in thick samples with higher axial resolution, we imaged every sample set with and without the cylindrical lens in place. Figure 4 is a three-dimensional voxel representation of detected RunX2 mRNA

in confluent MSCs 14 days post stimulation for both wide-field (green spheres) and astigmatic (red spheres) in the same image area. The nuclei are shown are blue and deconvolved using a GPU based algorithm<sup>35</sup> and we have omitted the cellular membrane for ease of visualization because the MSCs have already formed a three-dimensional matrix by 14 days. Using astigmatic imaging, we are able to detect more RunX2 mRNA with higher axial resolution. Spots that overlap in  $(x, y)$  are represented by yellow spheres and the size of sphere is directly proportional to the error in localization in the wide-field imaging mode. Figure 5 is a histogram of all detected RunX2 transcripts in confluent MSCs 14-days post stimulation, verifying that the image area shown in Figure 4 is not an anomaly and that we can detect more RunX2 mRNA with a higher axial resolution.

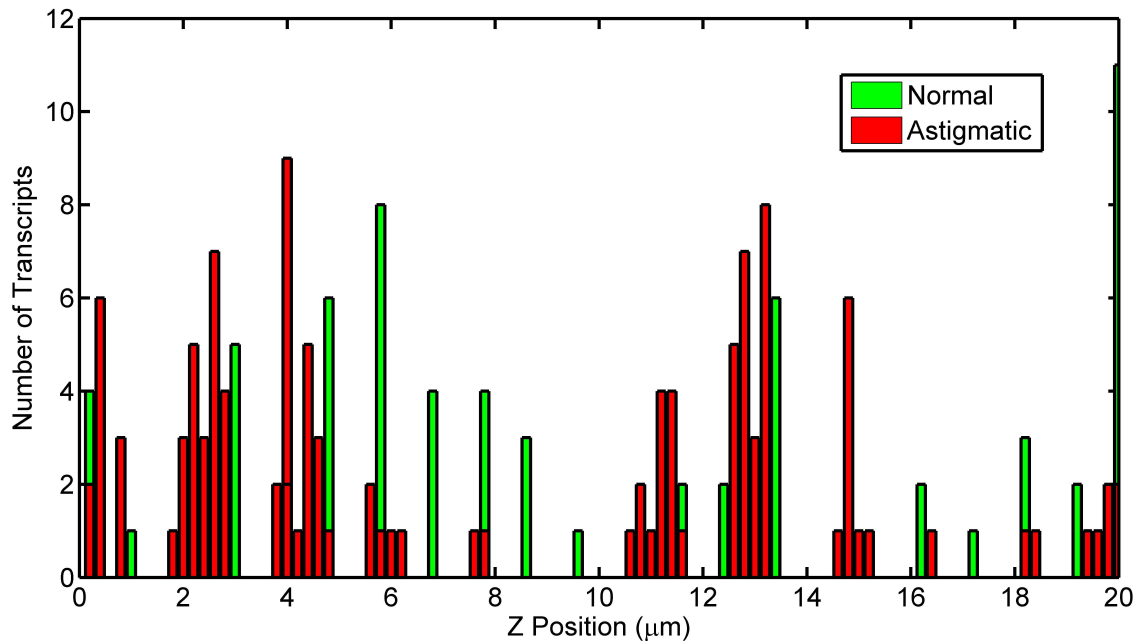


Figure 5. Histogram of axial ( $z$ ) positions of localized RunX2 mRNA in 14-day growth, confluent MSCs over 9 image areas. Wide-field imaging is shown in green and astigmatic imaging shown in red. There is clearly a difference in both the number of localized mRNA and axial distribution of localized mRNA between the two techniques. The astigmatic imaging has peaks that corresponds to the  $z$ -slices in the image stack. This is due to a higher likelihood of detecting an fluorescent spot near the focal plane because signal to noise drops as the PSF expands, which is especially pronounced for the astigmatic imaging microscope. Our fitting routine is less likely to find a spot that is above the photon threshold when it is far from the focal plane. This effect could be reduced by adding more  $z$ -slices, or by changing the optics to have less astigmatism, albeit at the expense of less  $z$ -localization accuracy.

#### 4. DISCUSSION

We find that astigmatic imaging leads to an increased number of detected smFISH spots and a more diverse distribution of  $z$ -positions when compared to standard wide-field imaging. An increase in detected transcripts can be attributed to the fact that astigmatic imaging provides high resolution in all three dimensions, whereas wide-field FISH imaging traditionally only offers only 2D resolution because a maximum intensity projection (MIP) is typically used during transcript counting.<sup>2,21</sup> For astigmatic FISH, transcripts that lie directly above or below other transcripts in the axial dimension are counted as separate while in wide-field imaging they would not be resolved due to the MIP processing step. As highlighted earlier this additional dimension of resolution is especially important with thick samples because the probability of overlapping transcripts in the axial dimension is greater. A more diverse distribution of transcripts in the axial dimension is seen in the MSC experiments (Figure 5) because the localization accuracy of astigmatic imaging is  $\sim 6\times$  greater and not limited to sampling from discrete focal planes as with wide-field imaging.

Our results for the digital quantity of RunX2 match with previous ensemble measurements during a similar experiment, showing a peak in RunX2 mRNA production around 14 days.<sup>33</sup> Of interest is the distribution of RunX2 mRNA at 0 days, which are fixed immediately following stimulation. Figure 6 shows that we are able to better capture the expression of RunX2 mRNA with our astigmatic FISH imaging, both detecting more mRNA and assigning those mRNA to a wider variety of cells because of the increased axial resolution. We find that wide-field FISH imaging fails to assign many mRNA to nuclei within the confluent MSC matrix, leading to an incomplete picture on when and in what quantity RunX2 mRNA are produced. This discriminatory ability is critical moving forward in understanding spatial and temporal dynamics of RunX2 mRNA expression during osteodifferentiation, because the three-dimensional matrix formed by MSCs becomes larger and more complicated as the process proceeds.

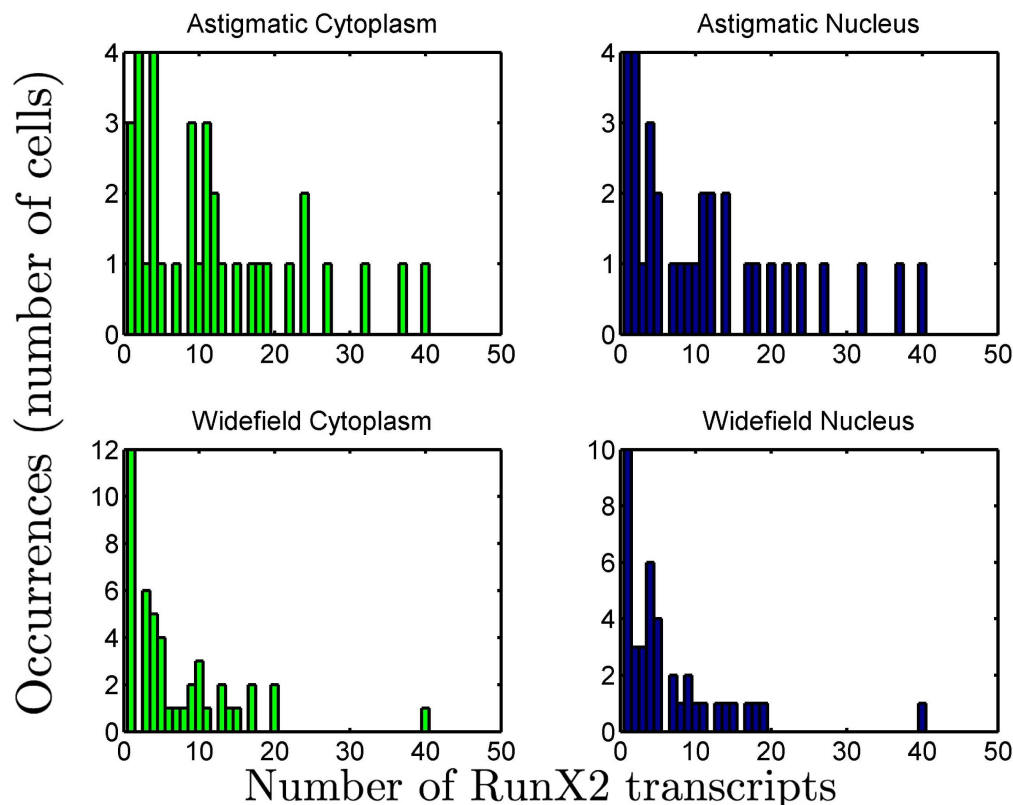


Figure 6. Histogram of the number of detected RunX2 mRNA for astigmatic FISH imaging (top row) and wide-field FISH image (bottom row) in 0-day growth, confluent MSCs over 9 images areas. While both sample sets show an exponential decay in the amount of detected transcripts, the distribution is more clearly filled in and separated between cells for the astigmatic imaging technique.

## 5. CONCLUSION

Quantitative imaging techniques are paving the way for a new understanding of biological processes at the single cell level. Many of these insights have been limited to axially thin samples, such as bacteria, non-confluent single cells, or tissue sections.<sup>2, 5, 7, 10, 16, 36–39</sup> Many interesting biological phenomena, such as osteodifferentiation, require imaging techniques capable of determining the number and location of individual bio-molecules (RNA transcripts, proteins, etc...) in thick samples that complicate both image acquisition and analysis. Here we have demonstrated an automated, multi-color microscope package capable of rapidly imaging and localizing individual

mRNA (labeled using smFISH) in axially thick samples by introducing an weakly defocusing cylindrical lens and utilizing GPU-based algorithms for spot finding, spot fitting, and image deconvolution.

The new capabilities of this microscope allow us to image RunX2 gene expression in confluent MSCs that have been stimulated to undergo osteodifferentiation. We find that in comparison to our previous wide-field microscope, the astigmatic imaging package provides higher spot finding efficiency and increased axial resolution at increased speeds. RunX2 mRNA is rapidly produced upon stimulation, with only a few cells continuing production past 14 days. Given the dependence of many downstream processes on RunX2 expression, one possible pathway to increase the yield of osteodifferentiation is control over the temporal duration of RunX2 transcript production.

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