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Methods for Assessing Nuclear Rotation and Nuclear Positioning in Developing Skeletal Muscle Cells

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Summary

Skeletal muscle cells are large syncytia, containing hundreds of nuclei positioned regularly along the length of the fiber. During development, nuclei are actively distributed throughout the myotube by the microtubule motor proteins, kinesin-1 and cytoplasmic dynein. Nuclear movement consists of translocation along the long axis of the cell concurrent with three-dimensional rotation of nuclei. In this chapter we describe methods for quantitatively assessing the speed of nuclear rotation in cultured myotubes using live-cell imaging techniques coupled with rigid-body kinematic analyses. Additionally we provide protocols for analyzing nuclear distribution in myotubes.

Keywords

Skeletal muscle; nuclear positioning; nuclear rotation; angular velocity; rigid body kinematics

1. Introduction

Early observation of myogenesis in primary chick and rat muscle cells revealed that nuclei in myotubes are highly mobile (1–3). In addition to making linear excursions through the cytoplasm, nuclei displayed prominent rotational dynamics. Rotation was observed in more than one plane, and nuclei were observed to rotate in both clockwise and counterclockwise directions; individual nuclei could also change their direction of rotation over time. Linear translocation of nuclei in cultured myotubes was observed at rates of up to 18µm/hour, with an average rate of ~6–9µm/hour (3), but only rough estimates of rotation rates were possible because this early work was imaged with phase contrast microscopy and thus only provided information in a single focal plane.

Advances in microscopy now allow the imaging of nuclear dynamics in three-dimensional space. Using confocal microscopy to obtain XY information in multiple Z-planes, we can visualize the entire nucleus as it rotates (4). Rotational dynamics are most clearly observed

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by imaging nuclei stained with fluorescent Hoechst dye, which binds DNA and brightly labels dense areas of heterochromatin. These chromocenters are distributed throughout the nucleus and retain their positions relative to one another as a nucleus translocates and rotates over short observation periods (minutes). Image analysis software can be used to track the movement of these chromocenters in three dimensional space over time. Using rigid-body kinematic analyses, we have developed protocols to quantitatively assess nuclear rotation (4).

Here, we describe methods for culturing C2C12 mouse myotubes and imaging myonuclear rotation. We include protocols for tracking chromocenters and provide a MATLAB-based algorithm that we developed to calculate the orientation and angular velocity of a nucleus in motion. In this algorithm the nucleus is analyzed as a rigid body; it is assumed that the chromocenters retain the same relative orientation within the nucleus as it undergoes motion. We have used these methods to show that nuclear rotation is abolished in the absence of microtubules, and severely compromised following siRNA-mediated reduction in kinesin-1 motor expression (4).

We have also shown that nuclear dynamics are necessary for proper distribution of nuclei throughout the developing myotube. Inhibition of rotation and translocation results in aggregation of nuclei (4,5), which is correlated with muscle dysfunction in mouse and fly muscles (6,7). Additionally, mispositioned nuclei are found in patients with centronuclear myopathies and Emery-Dreifuss muscular dystrophy (8,9). In recent years, significant progress has been made in understanding the mechanisms driving nuclear movement and distribution in developing muscle cells. In these studies, analyzing and graphically representing the distribution of nuclei in individual myotubes and in populations of myotubes following experimental perturbation is essential. Therefore, as an additional part of this chapter, we detail our methods for imaging whole myotubes, quantifying the position of the nuclei along the long-axis of these cells and effectively displaying distribution data for myotube populations. We have used these methods to show that loss of kinesin-1 motors from the nuclear envelope results in abnormal aggregation of nuclei at the midline of myotubes and that nesprins act as motor cargo adaptors for myonuclei (4,5).

Though we describe the culture and analysis of differentiated C2C12 myotubes, an immortalized mouse muscle cell line (10,11), we have found that the methods for analyzing nuclear rotation and distribution described here are also valid for primary myotube cultures. Furthermore, we propose that these methods may be effectively applied in the future to the analysis of nuclear rotation in other cell systems.

2. Materials

2.1 C2C12 Myotube Cell Culture

1. Mouse C2C12 myoblasts (CRL_1772) obtained from the American Type Culture Collection (ATCC). See Note 1.

¹Primary mouse myotube cultures can also be analyzed using these protocols. For further information on generating primary cultures, we refer the reader to the following references (12,13).

2. Dulbecco's modified Eagles's medium (DMEM)
3. Fetal bovine serum (FBS)
4. Horse serum
5. Stable l-glutamine supplement.
6. Growth Medium: DMEM supplemented with 10% (v/v) FBS, and 2 mM l-glutamine supplement.
7. Differentiation Medium: DMEM supplemented with 10% horse serum and 2mM l-glutamine supplement.
8. Trypsin solution: 0.25% trypsin
9. Glass-bottom dishes, 35 or 50mm, e.g. FluorDish (World Precision Instruments)
10. ACLAR embedding film (Ted Pella, Inc.) cut into squares that fit into the wells of 12-well culture dishes.
11. Collagen coating solution: Rat tail collagen, type 1 diluted to 50µg/ml in 0.02N acetic acid.
12. Dulbecco's phosphate buffered saline (DPBS)
13. Incubator at 37°C with 5% CO₂.
14. Inverted light microscope.
15. Benchtop centrifuge.

2.2 Cell Transfection Reagents

1. Lipofectamine 2000 transfection reagent (Life Technologies, 11668-027).
2. Unsupplemented DMEM.
3. Mammalian expression construct for EGFP.

2.3 Live-cell microscopy for nuclear rotation analysis

1. Spinning disk confocal fluorescence microscope equipped with a motorized stage, 60× and 100× oil-immersion objectives, EMCCD camera, Volocity 3D Image Analysis Software (Improvision) and if available, a perfect focus system (e.g. Perkin Elmer Ultraview Vox Spinning Disk confocal with a Nikon Ti Microscope equipped with 60x/1.49 NA and 100x/1.49 NA oil-immersion apochromatic objectives (Nikon), and a Hamamatsu EMCCD C9100-50 camera).
2. Microscope environmental chamber maintained at 37°C and 5% CO₂ (CO₂ control is optional for imaging less than ~5 hrs).

3. Imaging Media: phenol-red free DMEM, high glucose with 25 mM HEPES, supplemented with 10% horse serum and 2 mM l-glutamine supplement.
4. Live cell Hoechst dye (Hoechst 33342, trihydrochloride, trihydrate, FluoroPure grade)(0.5 µg/ml final concentration).
5. Mineral oil.
6. Volocity 3D tracking software protocol described below.
7. MATLAB nuclear rotation analysis algorithm included below.

2.4 Cell Fixation and Immunofluorescence

1. Phosphate-buffered saline (PBS): 1.3 M NaCl, 70 mM Na₂HPO₄•2H₂O and 30 mM NaH₂PO₄•H₂O in ddH₂O. Adjust pH to 7.2.
2. 4% paraformaldehyde in PBS, warmed to 37°C.
3. 0.1% Triton X-100 in PBS.
4. Bovine serum albumin
5. Goat serum
6. Blocking solution: 1× PBS supplemented with 1% BSA, 5% goat serum (filtered and stored at 4°C)
7. Anti-alpha-actinin primary antibody (clone EA-53, mouse, Sigma-Aldrich), 1:500 dilution.
8. Anti-alpha-tubulin primary antibody (YL1/2, rat, AbD Serotec), 1:500 dilution.
9. Goat anti-Mouse IgG (H+L) Secondary antibody, Alexa Fluor 488 conjugate (Life Technologies, A-11001), 1:500 dilution.
10. Goat anti-rat IgG (H+L) Secondary antibody, Alexa Fluor 594 conjugate (Life Technologies, A-11007), 1:500 dilution.
11. Hoechst dye (Hoechst 33342, see above), 1:500 dilution
12. Antifade mounting media, e.g. ProLong Gold Antifade (Life Technologies, P36930)
13. 40×22 mm (#1.5) glass coverslips.

2.5 Fixed Cell Microscopy for Nuclear Distribution Analysis

1. Spinning disk confocal fluorescence microscope equipped with a motorized stage, 40× oil-immersion objective, EMCCD camera and Volocity 3D Image Analysis software (e.g. Perkin Elmer Ultraview Vox Spinning Disk confocal with a Nikon Ti Microscope equipped with a motorized stage, 40x/1.30 NA oil-immersion apochromatic objective

(Nikon), Hamamatsu EMCCD C9100-50 camera and Volocity 3D Image Analysis software (Improvision/Perkin Elmer)).

2. Microsoft Excel.
3. GraphPad Prism (GraphPad software).

3. Methods

3.1 C2C12 Myotube Culture Protocols

1. Culture proliferating C2C12 myoblasts in growth medium: DMEM + 10% FBS + 2mM l-glutamine supplement. Myoblasts may be cultured on plastic culture dishes. To split, wash cells once with DPBS, add 0.25% trypsin and incubate until cells release from the dish, add growth medium and spin cells at 1000 rpm for 2 min in a 15ml conical tube to pellet. Gently resuspend cells in 1 ml of fresh growth medium before diluting and plating cells on 10 cm culture dishes in additional growth medium. See Note 2.
2. For myotube cultures, myoblasts must be plated in growth medium on collagen-coated glass-bottom dishes (for live cell imaging or immunofluorescence) or on collagen-coated ACLAR plastic embedding film coverslips in 12-well plates (for immunofluorescence). See Notes 3 & 4.

3. To coat plates with a thin layer of collagen:

Dilute stock collagen solution to 50 µg/ml using 0.02N acetic acid.

Add diluted collagen solution to dishes as follows:

1–2 ml to the glass center of a 35 mm dish

2–3 ml to the glass center of a 50 mm dish

1 ml/well on a 12-well dish containing ACLAR coverslips (See Note 4)

Incubate at room temperature for one hour in the hood.

²To maintain proliferating C2C12 myoblasts, cells must be split regularly at sub-confluency, before the cells commit to differentiation. As the cells become increasingly confluent, they will begin to elongate and align with one another, thereby decreasing the myoblast population capable of further proliferation. Even with strict splitting habits, the differentiation potential of these cells typically decreases with passage number, therefore, it is best to thaw a new vial of myoblasts after ~10 passages, or when you start to notice a decline in myotube formation. Upon obtaining a vial of C2C12 myoblasts, it is advisable to initially expand the culture and freeze down stock vials of low-passage number cells at high density.

³Plates and ACLAR coverslips can also be coated with other substances, including gelatin or laminin (14), a fibroblast substratum (15), or matrigel (16).

⁴ACLAR plastic embedding film is used in place of glass coverslips because it was noted that the myotubes readily pull off of the glass surface when they begin to spontaneously contract. However, it has been our experience that C2C12 cells grow well on one side of the ACLAR film but pull into abnormal piles of cells when cultured on the opposite side. We have not been able to distinguish which is the “correct” side visually, therefore, it is important to test pieces of ACLAR in both orientations by growing cells on each side and keeping careful track of the “correct” side of the film. It will be necessary to test each new sheet of ACLAR in this manner. Sterilize scissors prior to cutting ACLAR and work in the hood.

Aspirate the remaining solution.

Rinse well with DPBS to remove the acid.

Air-dry plates in the hood, exposed to UV for 10 minutes.

Plates may be used immediately or stored at 4°C under sterile conditions.

We have stored plates for up to 3 weeks without adverse effects on myotube growth or morphology.

4. To induce differentiation of myoblasts to myotubes, switch to differentiation medium when myoblasts are ~70% confluent. Cells will continue to divide for a time after switching to differentiation medium and if they are too confluent when put into differentiation medium, they will likely overgrow and die rather than differentiate.
5. Differentiation medium should be replaced daily after induction to replenish nutrients, remove cell debris and maintain pH. Typically, cells will begin to fuse ~2–3 days after induction of differentiation. Multinucleated myotubes will increase in size and maturity over the next 5 days. In more mature cultures, myofibril twitching can be observed as well as spontaneous contraction of whole myotubes. As cultures get older, >7 days post-differentiation, significant branching of myotubes can be observed, sometimes creating large networks of fused myotubes. At this stage, the health of the culture begins to decline compromising further analyses of nuclear dynamics and distribution. Typically, analysis of myotubes is performed 6–7 days post-induction of differentiation.

3.2 C2C12 Transfection (Optional, see Note 5)

1. Transfect differentiating myotubes with EGFP plasmids using Lipofectamine 2000 reagents according to the manufacturer's protocol. Transfections have been effective on day 4 and 5 following induction of differentiation (48–72 hrs prior to fixation or live cell analysis).

3.3 Live-Cell Imaging for Analysis of Nuclear Rotation

1. Aspirate differentiation medium from the glass-bottom dish and rinse myotubes with pre-warmed (37°C) phenol-red free imaging media.
2. Add pre-warmed imaging medium containing 0.5µg/ml Hoechst dye to dish, overlay the medium with warm mineral oil, and place the dish in the

⁵Although it is not necessary, transfecting the cells with DNA constructs for EGFP or other cytosolic or plasma membrane-bound fluorescent protein makes it easier to distinguish myotube boundaries, myotube branching and relative position of nuclei within the live myotubes. In the absence of such a marker, it is important to become adept at recognizing nuclei present within myotubes compared with nuclei in mononucleated myocytes, which can often lie beneath myotubes on the culture plate. However, if it is necessary to know where the nuclei lie within the myotube and/or that all nuclei being analyzed are in the same myotube, expression of EGFP will aid in delineating the boundaries of the myotube.

microscope environmental chamber to incubate for 20 minutes prior to the start of imaging. See note 6.

3. Locate myotube nuclei to image. Nuclei in myotubes can be distinguished from myoblast nuclei by their shape and pattern of heterochromatin. Nuclei in myoblasts are typically flatter, wider and have more numerous and smaller chromocenters. In comparison, nuclei in myotubes are more spherical, have a greater z-depth and fewer, larger chromocenters. One can often distinguish myotube nuclei within the same myotube by looking for a line of nuclei sharing this appearance. See Figure 1. Additionally, if myotubes have been transfected with EGFP, this signal can be used to identify myotubes and associated nuclei. See Note 5.
4. Using a 60× or 100× objective, capture images of nuclei over time using the following guidelines:
 - a. Obtain z-series encompassing the entire depth of the myotube (~15–40µm) with a 0.5 µm step size. See Note 7.
 - b. Take images at a rate of 1 z-series per minute for at least 15 minutes (16 timepoints). See Figure 2A.
 - c. Adjust exposure time, gain and offset settings to obtain quality images while minimizing myotube/nuclei exposure to laser light. If EGFP is used to identify myotubes, it is best to only take one z-series in the 488 channel at the start or end of the time-series. See Note 8.
 - d. If available, a perfect focus system on the microscope will help to minimize axial focus fluctuations, thereby improving the quality of the data.
5. Continue imaging nuclei in additional myotubes on this plate as needed. Switch to a new plate of cells after 2–3 hours, or if the health of cells begins to decline.

3.4 Nuclear Rotation Analysis

In this analysis, the chromocenters within a nucleus are first tracked in XYZ over time in Volocity 3D image analysis software. The resulting tracking data is used to calculate the orientation and angular velocity of rotation using rigid body kinematic analyses performed

⁶The use of HEPES buffered medium will help maintain pH in the absence of the 5% CO₂ atmosphere. We have imaged myotube cultures up to ~5 hours without noticing alterations in myotube health or changes in nuclear dynamics. Overlaying the imaging media with mineral oil reduces evaporation, thereby preventing changes in osmolarity. Note that it is difficult to sufficiently remove the mineral oil following imaging if it is necessary to fix and stain these myotubes for further analysis. Alternatively, the environmental chamber may be humidified during imaging.

⁷We have used z-step sizes as small as 0.2 µm. We have found that step sizes smaller than 0.5 µm only marginally improve precision of chromocenter tracking in the z-dimension. The 0.5 µm z-step size minimizes exposure time while still allowing for reliable chromocenter tracking and subsequent assessment of angular velocity.

⁸When the myotubes are exposed to excessive laser light, they may begin to contract forcefully, which can cause the myotube to detach from the plate. This is typically rare during a 15 min time series, but becomes more likely as the length of time series increases and when EGFP is used to identify myotubes.

in MATLAB. The nucleus is analyzed as a rigid body where it is assumed that the chromocenters retain the same relative orientation in the nucleus as it undergoes motion. A minimum of three chromocenters (spatial points) are required for the calculation where each track contains the time dependent coordinates of a single chromocenter at each time point. More than three points can be used and in general will result in more accurate results for the spatial orientation. The shape of the nucleus also deforms to some degree during its course of travel and the tracking of chromocenters has a finite error. However the rigid body analysis performed here is still applicable, and the error introduced by these factors can be averaged out through the use of several tracking points.

1. Open the file containing the time series images of nuclei rotating in Volocity 3D image analysis software.
2. Open the first captured time series and crop the images in XY to include only one nucleus of interest. Make sure to advance through the time points and verify that the cropped region is large enough to encompass all linear movement of the nucleus over time. See Note 9.
3. In the Volocity Measurements View, set up the following protocol to identify and track the bright Hoechst dye-labeled chromocenters in XYZ over time:
 - a. Find Objects – Define the Threshold (~3–5%) and the Minimum Object Size (~0.18–0.2 μm^3)
 - b. Remove noise from objects – medium filter
 - c. Separate touching objects – 0.5 μm^3

The software will identify chromocenters in the ROI, both those in the nucleus of interest and those in any other nuclei present in the ROI. Advance through each frame of the time series to verify that the program is correctly identifying chromocenters. Adjust the threshold and minimum object size as needed to achieve optimal identification. While a minimum of three chromocenters is necessary for angular velocity calculations, additional points in the nucleus will improve the rotation data. We have found that the largest chromocenters are tracked most accurately in XYZ space.

- d. Track Objects – choose the following parameters: shortest path, ignore new objects, set the maximum distance between objects. Verify by eye that the tracks are correct by playing the time series through. Check that the software

⁹It is not necessary to crop the images for the analysis, but by eliminating the image data around a specific nucleus, we have noticed that Volocity more effectively finds chromocenters in successive frames and creates valid tracks.

is able to track each individual chromocenter continuously from the first frame to the last frame. Alter the parameters above as necessary to achieve accurate tracks. See Note 10 and Figure 2.

4. Even with significant optimization, it may not be possible to achieve complete, correct tracks for all chromocenters in a nucleus. If this is the case, in the measurements window, filter the track data by time span and eliminate data for all tracks shorter than the duration of the time series (highlight a track in the measurements window and press delete). Additionally, remove any data for chromocenters/tracks that are not in the nucleus of interest. Sort the data by Track ID in the Measurement Table.
5. In the Measurements tab, choose Columns. In the opened window, define which measurements to include in the data file. Include Object ID, Track ID, Timepoint, Relative Time (s), Centroid X (μm), Centroid Y (μm), Centroid Z (μm). Other data may also be included in the data file, but are not necessary for angular velocity analysis.
6. In the Measurements tab, choose Make Measurement Item. Name the Measurement Item as desired, all of the data will now appear in a spreadsheet in the Volocity library. Additionally, export this table as a .CSV file.
7. Open the MATLAB script entitled *ChromocenterDataParse.m*. When prompted enter the name of the .CSV file containing the tracking data. This script parses the Volocity data into MATLAB. Refer to Note 11 for source code listing.
8. To calculate the total angular speed, run *NucleusAngularVec.m*. Refer to Note 12 for source code listing and a detailed explanation of the algorithm.
9. Repeat steps 1–8 for each nucleus to be analyzed.

3.5 Cell Fixation and Immunostaining for Analysis of Nuclear Distribution

1. Pre-warm 4% PFA in PBS to 37°C.
2. Aspirate differentiation medium from the 12-well plate with ACLAR coverslips and rinse the myotubes with warm DPBS.
3. Add warm 4% PFA to fix the cells and incubate at room temperature 10 minutes.

¹⁰Initially, try using the Volocity command “estimate maximum distance between objects automatically”. This will provide a guideline for defining this distance parameter manually in subsequent analysis. If a number is manually entered, the software will report the same tracks in repeated analyses of the same chromocenter, however, if it estimates automatically, the tracking data will be slightly different in repeated analyses.

¹¹ChromocenterDataParse.m source code

4. Aspirate the 4% PFA and wash the coverslips with PBS (3×5 min) at room temperature.
5. Permeabilize the cells by incubating with 0.1% Triton X-100 in PBS for 5 min.
6. Wash the cells 3×5 min with PBS.
7. Incubate fixed myotubes for 1 hour in blocking solution at room temperature.
8. Incubate myotubes for 2 hours with the anti-alpha-actinin antibody (1:500) and anti-alpha-tubulin primary antibody (1:500) in blocking solution at room temperature. See Note 13.
9. Wash 3×10 min with PBS.
10. Incubate myotubes for 1–2 hours with Alexa 488 conjugated anti-mouse secondary antibodies (1:500), Alexa 594 conjugated anti-rat secondary antibodies (1:500) and Hoechst dye (0.5 $\mu\text{g}/\text{ml}$) in blocking solution at room temperature.
11. Wash 3×10 min with PBS.

```

% ChromocenterDataParser.m
% Centroid for each chromosome and time for each track is read into
% xCent yCent zCent time matrices
%
% Open CSV file containing track information
[filename, pathname]=uigetfile('*.csv','Select a CSV file');
fid=fopen(fullfile(pathname,filename));

tline=fgetl(fid); %First line is a space
tline=fgetl(fid); %Second line are labels
labelread=textscan(tline,'%s','Delimiter',' ');
labels=labelread(1); %Cell array of column labels

clear xCent yCent zCent time tracks dataCnt
trackCnt=0; dataCnt=0; tracks=0;

tline=fgetl(fid);
while(tline~-1)
    cnt=1; cflag=0;
    while(tline)
        label=stok(labels(cnt),' ');
        [data tline]=strtok(tline,' ');

        if(strcmp(label,'Type')) %Don't read Track Objects
            if(strcmp(data,'Track'))
                tline=fgetl(fid);
                cflag=1;
                break;
            end
        end
        if(strcmp(label,'Rel. Time (s)'))
            RelTime=str2double(strtok(data,' '));
        end
        if(strcmp(label,'Centroid X (um)'))
            xc=str2double(strtok(data,' '));
        end
        if(strcmp(label,'Centroid Y (um)'))
            yc=str2double(strtok(data,' '));
        end
        if(strcmp(label,'Centroid Z (um)'))
            zc=str2double(strtok(data,' '));
        end
        if(strcmp(label,'Track ID'))
            trackID=str2double(strtok(data,' '));
        end

        cnt=cnt+1;
    end
    if(cflag) continue; end;
    tCID=find(tracks==trackID);
    if(tCID)
        dataCnt(tCID)=dataCnt(tCID)+1;
    else
        trackCnt=trackCnt+1;
        tCID=trackCnt;
        tracks(tCID)=trackID;
        dataCnt(tCID)=1;
    end
    time(tCID,dataCnt(tCID))=RelTime;
    xCent(tCID,dataCnt(tCID))=xc;
    yCent(tCID,dataCnt(tCID))=yc;
    zCent(tCID,dataCnt(tCID))=zc;

    tline=fgetl(fid);
end
fclose(fid);

```

¹²NucleusAngularVec.m source code

12. Mount ACLAR coverslips either on a glass slide or on a 22×40 mm glass coverslip (#1.5) with antifade media. See Note 14.
13. Let antifade cure overnight at room temperature before imaging.

```

%NucleusAngularVel.m
%This algorithm calculates the euler angles at each time step
%from the centroid data loaded by the dataParse routine.
%It outputs angular velocity
%
%It uses xCent,yCent,zCent and time from ChromocenterDataParse.m

Nt=size(xCent,2); % number of time points
Np=size(xCent,1); % number of track points

for t=2:Nt
    %Calculate Centroids
    v1c=[0;0;0]; v2c=[0;0;0];
    for ii=1:Np
        v1c=v1c+(xCent(ii,t-1);yCent(ii,t-1);zCent(ii,t-1));
        v2c=v2c+(xCent(ii,t);yCent(ii,t);zCent(ii,t));
    end
    v1c=v1c/Np; v2c=v2c/Np;
    %Calculate Correlation matrix
    c=zeros(3,3);
    for ii=1:Np
        vec1=[xCent(ii,t-1);yCent(ii,t-1);zCent(ii,t-1)]-v1c;
        vec2=[xCent(ii,t);yCent(ii,t);zCent(ii,t)]-v2c;
        c=c+vec2*vec1';
    end
    c=c/Np;
    %Calculate Rotation Matrix
    [u,v,w]=evec(c);
    R=[u v w; 0 0 0; 0 0 0]; % R=[u v w; 0 0 0; 0 0 0]
    %Get Euler Angles from Rotation Matrix
    theta=atan2(R(3,2),R(3,1));
    psi=atan2(R(3,2)/cos(theta),R(3,1)/cos(theta));
    phi=atan2(R(2,1)/cos(theta),R(1,1)/cos(theta));

    %Get angular velocity
    disp(R);
    disp([theta psi phi]);
    dt=time(1,t)-time(1,t-1);
    %Angular velocity of each Euler component
    wv(t,1)=(theta psi phi)/dt;
    %Resultant angular velocity (angular speed)
    wc(t,1)=norm([theta psi phi])/dt;
    %Translation vector of nucleus from previous time step
    %Not used here - can be used to determine linear velocity,
    %acceleration, etc...
    translation(t-1,t)=v2c-R*v1c;
end

str=fprintf('Time Average Angular velocity (total) %0.5g degrees per\n',wc);
disp(str);
str=fprintf('Time Average Angular velocity (pitch) %0.5g degrees per\n',wv(1,1));
disp(str);
str=fprintf('Time Average Angular velocity (yaw) %0.5g degrees per\n',wv(2,1));
disp(str);
str=fprintf('Time Average Angular velocity (roll) %0.5g degrees per\n',wv(3,1));
disp(str);

figure;
hold off;
plot(time(1,1):(Nt-1)),wc*180/pi,'k','linewidth',3);
hold on;
plot(time(1,1):(Nt-1)),wv(1,1)*180/pi,'r','linewidth',1);
plot(time(1,1):(Nt-1)),wv(2,1)*180/pi,'b','linewidth',1);
plot(time(1,1):(Nt-1)),wv(3,1)*180/pi,'g','linewidth',1);

legend('Total Angular Velocity','Pitch Angular Velocity','Yaw Angular Velocity','Roll\nAngular Velocity');
xlabel('Time (seconds)');
ylabel('Angular Velocity (degrees per second)');

```

To begin the tracking analysis we sample a set of N_p points (XYZ centroid of chromocenters) on the nucleus in three dimensional space at a set of discrete times $t(n)$. The set of points at one time epoch n are recorded as column vectors. This data is retrieved from the Velocity 3D image analysis software (Section 3.4)

$$\mathbf{x}(n) = \begin{bmatrix} x_1(n) \\ x_2(n) \\ \vdots \\ x_{N_p}(n) \end{bmatrix}, \mathbf{y}(n) = \begin{bmatrix} y_1(n) \\ y_2(n) \\ \vdots \\ y_{N_p}(n) \end{bmatrix}, \mathbf{z}(n) = \begin{bmatrix} z_1(n) \\ z_2(n) \\ \vdots \\ z_{N_p}(n) \end{bmatrix}$$

The centroid of the set of points is then calculated through averaging. Note this is the centroid of the set of chromocenters of a single nucleus. Section 3.4 refers to finding the centroid of single chromocenter.

$$x_c(n) = \frac{1}{N_p} \sum_{m=1}^{N_p} x_m(n), y_c(n) = \frac{1}{N_p} \sum_{m=1}^{N_p} y_m(n), z_c(n) = \frac{1}{N_p} \sum_{m=1}^{N_p} z_m(n)$$

Next a vector $\mathbf{v}_m$ from each point on the body to the centroid of the set of points at times n and $n-1$ is calculated. The vector $\mathbf{v}_m$ is then multiplied by its transpose from the previous time step and summed over all points to calculate the correlation matrix, $\mathbf{C}$ (17).

3.6 Imaging Myotubes for Analysis of Nuclear Distribution

1. Before starting to obtain images, calibrate the motorized stage on the microscope.

$$\mathbf{v}_m(\mathbf{n}) = \begin{bmatrix} x_m(n) - x_c(n) \\ y_m(n) - y_c(n) \\ z_m(n) - z_c(n) \end{bmatrix}, \mathbf{v}_m(\mathbf{n}-1) = \begin{bmatrix} x_m(n-1) - x_c(n-1) \\ y_m(n-1) - y_c(n-1) \\ z_m(n-1) - z_c(n-1) \end{bmatrix}$$

$$\mathbf{C} = \frac{1}{N_p} \sum_{m=1}^{N_p} \mathbf{v}_m(\mathbf{n}) \mathbf{v}_m(\mathbf{n}-1)^T$$

The 3×3 correlation matrix can then be used to calculate the rotation matrix between the set of points at times n and $n-1$ using singular value decomposition. The singular value decomposition of the correlation matrix $\mathbf{C}$ results in $\mathbf{C} = \mathbf{U}\mathbf{\Sigma}\mathbf{V}^*$. Following the singular value decomposition the rotation matrix can be calculated as

$$\mathbf{R} = \mathbf{U} \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & |\mathbf{UV}^*| \end{bmatrix} \mathbf{V}^*$$

Using the components of the rotation matrix the Euler angles can be calculated. We adopted the convention where:

$$\mathbf{R}_x(\psi) = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \cos(\psi) & -\sin(\psi) \\ 0 & \sin(\psi) & \cos(\psi) \end{bmatrix}$$

$$\mathbf{R}_y(\theta) = \begin{bmatrix} \cos(\theta) & 0 & \sin(\theta) \\ 0 & 1 & 0 \\ -\sin(\theta) & 0 & \cos(\theta) \end{bmatrix}$$

$$\mathbf{R}_z(\phi) = \begin{bmatrix} \cos(\phi) & -\sin(\phi) & 0 \\ \sin(\phi) & \cos(\phi) & 0 \\ 0 & 0 & 1 \end{bmatrix}$$

$$\mathbf{R} = \begin{bmatrix} R_{11} & R_{12} & R_{13} \\ R_{21} & R_{22} & R_{23} \\ R_{31} & R_{32} & R_{33} \end{bmatrix} = \mathbf{R}_z(\phi) \mathbf{R}_y(\theta) \mathbf{R}_x(\psi)$$

Solving for the Euler angle θ , ψ , and ϕ we get

$$\theta = -\sin^{-1}(R_{31})$$

$$\text{if } R_{31} \neq \pm 1 \text{ then } \psi = \text{atan2}\left(\frac{R_{32}}{\cos\theta}, \frac{R_{33}}{\cos\theta}\right) \text{ else } \psi = \text{atan2}(R_{32}, R_{33})$$

$$\text{if } R_{31} \neq \pm 1 \text{ then } \phi = \text{atan2}\left(\frac{R_{21}}{\cos\theta}, \frac{R_{11}}{\cos\theta}\right) \text{ else } \phi = 0$$

The angular velocity is then calculated as the time rate of change of the Euler angles and the total angular speed is calculated from the resultant vector

$$\boldsymbol{\omega} = \frac{[\theta, \psi, \phi]}{\Delta t} \text{ and } \omega_t = |\boldsymbol{\omega}|$$

2. Using a 40× objective, capture tiled images of large regions of fixed immunostained myotubes with Volocity using the following guidelines:
 - a. Define the region of interest (ROI) to be captured on the grid in the XY Stage view. Typically a region of $\sim 1000 \mu\text{m} \times 1000 \mu\text{m}$ is appropriate. Volocity will acquire the minimum number of fields to completely cover the ROI. A ROI $\sim 1000 \mu\text{m}^2$ is typically achieved by a 4×4 grid of fields or tiles (16 tiles) with 10% overlap at 40x. See Figure 3 and Note 15.
 - b. Find the approximate center focal plane of all of the myotubes in the current field of view. Set this position as zero. Set the image acquisition parameters to obtain a z-series for each field of view, starting $\sim 20 \mu\text{m}$ below and moving to $\sim 20 \mu\text{m}$ above this center point, with a 1–2 μm z-step size. The size of this z-stack should encompass the entire depth of all of the myotubes in the field of view (typically $\sim 40 \mu\text{m}$).
 - c. Set the software to obtain images in the appropriate channels for Hoechst dye, alpha-actinin/Alexa 488, and tubulin/Alexa 594 and adjust exposure settings as needed to obtain quality images.
 - d. Set the software to save all raw tiles. You can choose to either stitch the tiles together immediately after obtaining the images or to stitch at a later time. Stitching large regions can take significant amounts of time, so offline stitching is recommended. In Volocity V6.0 or higher, select Stitch Images in the Tools menu, in the pop-up window, choose “Create a Stitched Image” and click to option “Correct for Brightness”.
3. Use the grid on the XY stage view to define the next ROI. To avoid imaging the same region twice, it is advised to define ROIs in a sequential pattern. If the myotube cultures have differentiated and fused well, 9 to 10

To calculate the total angular speed a MATLAB script was developed which incorporates the equations as described in the previous steps. First, line 6 of the MATLAB script ChromocenterDataParse.m is modified so that it opens the CSV output from Volocity and it is run. This script parses the Volocity data into MATLAB. Next NucleusAngularVec.m is run to calculate the total angular speed.

¹³Immunostaining for alpha-actinin is an effective way to identify the ends of the myotubes in the culture dish. Phase contrast images would also be appropriate, if this is an option on the microscope. Immunostaining for beta-tubulin aids in assessing whether a given nucleus is within the myotube.

¹⁴It is possible to image the myotubes through the ACLAR plastic film, but we have had more success mounting the ACLAR pieces on glass coverslips and imaging through the glass instead. Adjustable stage adaptors accommodate 40mm glass coverslips.

¹⁵If the myotubes have been transfected with a tagged protein of interest, but the numbers of myotubes expressing the transgene are small and it is only these transfected myotubes that are to be part of the nuclear distribution analysis, it may be better to define smaller ROIs that encompass only a single transfected myotube (or a few if they are close together). In this situation, taking large, unbiased ROIs may result in substantial unusable data if they lack any myotubes expressing the transgene. In this case, move systematically through the coverslip to obtain images of all of the expressing-myotubes present on the coverslip, or until the pre-defined number of myotubes has been obtained.

ROIs are typically sufficient to obtain nuclear distribution data for 50–60 myotubes.

3.7 Analysis of Nuclear Distribution

1. Create a maximum projection of a stitched ROI in Volocity. Include the Hoechst dye, alpha-actinin and tubulin channels in the image. See Figure 4A and Note 16.
2. In the following analysis, only include myotubes that lie fully within the stitched ROI. Do not include branched myotubes in the analysis.
3. In the Volocity Measurements View, starting at the bottom and/or left end of a given myotube, as shown by the alpha-actinin staining, use the measurement tools to determine the X,Y coordinates of the myotube end (e1)(Figure 4A,B). These coordinates can either be determined in pixels or preferably in microns if the measurement tools are calibrated to the objective.
4. Determine the X,Y coordinates of the centroid of each nucleus in the myotube, moving sequentially from the left of the myotube to the right (or the bottom to top, if the myotube is oriented vertically). Assign sequential numbers to each nucleus (Figure 4A,B).
5. Determine the X,Y coordinates of the right (or top) end of the myotube (e2).
6. Repeat this process for all of the myotubes in each stitched ROI.
7. Using the X,Y coordinates for each myotube end, determine the length of each myotube using the distance equation: $d = \sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2}$
8. Using e1 and e2 to define a vector, $\vec{v_e}$ project the X,Y coordinates of the centroid of each nucleus along a perpendicular path onto the line segment (Figure 4C). Assuming $\vec{v_3}$ is the vector from e1 to the centroid of a nucleus labeled 3, the distance d from e1 to projected point on the line is $d = \left\| \frac{\vec{v_e} \cdot \vec{v_3}}{\|\vec{v_e}\|^2} \vec{v_e} \right\|$. After this projection, all of the centroids of each nucleus lie along the line segment extending between myotube ends (Figure 4D).
9. Determine the distance between adjacent nuclei on this line segment.

¹⁶Although the distribution of nuclei is analyzed in the maximum projection image, it is important to refer to the original images in the z-series when there is question as to whether a given nucleus is within the myotube of interest. As discussed previously, often myoblasts will be below myotubes and in a maximum projection, it may not be immediately clear that the myoblast nucleus is in a separate cell. Along with using the shape and chromocenter pattern in a nucleus to assess whether it is within the myotube, it is often helpful to move through the z-series plane-by-plane or view the stack in an X,Z or Y,Z orientation. Myoblast nuclei will almost always appear in a z-plane below the myotube nuclei and will likely not be in a plane with significant alpha-actinin signal. Furthermore, the absence of signal for alpha-actinin or beta-tubulin where the nucleus resides also typically indicates the nucleus of interest is within the myotube.

- 10.** To visually represent the position of nuclei in individual myotubes within a population of myotubes, organize your data to make a plot of nuclear distribution using Graphpad Prism software (See Figure 4E).
- a.** For each myotube, set the midpoint between e1 and e2 to zero and determine the distance of each nucleus (and myotube ends) relative to this mid-point, with nuclei to the left of the midline being assigned negative values and the nuclei to the right of the midline assigned positive values.
 - b.** Number the myotubes in a population according to length, setting the longest myotube equal to one, second longest equal to 2, and so on.
 - c.** Create an X,Y graph in Prism in which the number of the myotube (Y value) is assigned to all points in the myotube, including e1, e2 and each nucleus (X values).
 - i.** In Prism make the following selections:
 - 1.** Make New Data Table (+ Graph)
 - 2.** Choose X,Y graph and Enter and plot a single Y value for each point
 - ii.** In the table created, enter the distance data for myotube ends and centroid of each nucleus, as calculated in 10a, into the X column. Group this data by myotube, and organize the myotubes in the column according to myotube length, starting with the longest myotube.
 - iii.** In the first Y column, enter the number of the myotube, as determined in 10b, for all of the X values corresponding to myotube ends (i.e. the first and last data point in each myotube).
 - iv.** In the second Y column, enter the number of the myotube, as determined in 10b, for all of the X values corresponding to nuclei.
 - v.** In the graph that is produced, change the color or shape of values from the first Y column to delineate that they are myotube ends.

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Figure 1. Examples of myoblast and myotube nuclei labeled with Hoechst dye
Hoechst dye binds DNA and brightly labels dense areas of heterochromatin (chromocenters). The nucleus in a mononucleated myoblast (MB) is typically flatter, wider and has smaller and more numerous chromocenters. The nuclei in myotubes (MT) are usually more spherical, have fewer, larger chromocenters and are often found in a line along the long-axis of the myotube (two-headed arrow). Scale = 10 μ m.

Figure 2. Example of nuclear rotation and chromocenter tracking

(A) DNA was labeled with Hoechst dye and a nucleus within a myotube was imaged at a frame rate of 1 frame per minute for 15 minutes. Maximum projections of confocal z-stacks are shown over time. Three of the chromocenters are highlighted to aid in visualization of rotation. Scale bar: 2 μ m. (B) Chromocenters were tracked in X, Y and Z over time using Volocity image analysis software and the resulting 2D projection of the tracks is shown. Black tracks correspond to the highlighted chromocenters in panel A. The nucleus rotates while translocating to the left. Applying the algorithm included in the *NucleusAngularVec.m* MATLAB script, the mean total angular velocity of this nucleus is 4.8°/minute. The nucleus rotates 72° in 15 minutes.

Figure 3. Guideline for imaging large regions of myotubes

Using a 40× objective, capture a ROI of ~1000μm². This is typically composed of 16 tiles with 10% overlap. Each of these tiles is composed of a z-stack encompassing a depth of ~40μm. To avoid imaging the same region more than once, use the grid pattern in the Volocity XY stage view to define ROIs, taking care to move across and down the coverslip in a sequential pattern.

Figure 4. Myotube nuclear distribution analysis

(A) A small region of a stitched ROI shows C2C12 myotubes and surrounding myoblasts. Myotubes were immunostained α -actinin (green) and α -tubulin (red) and DNA was stained with Hoechst dye (blue). Image is a maximum projection of confocal z-sections. This region includes two complete myotubes suitable for nuclear distribution analysis (a & b, outlined for clarity, nuclei within the myotubes are circled). This region also includes numerous myoblasts and additional myotubes extending out of the shown region. Scale = 50 μ m. (B) Illustration of the two myotubes in (A); nuclei in each myotube are numbered and a line segment between the ends (e1 and e2) of the myotubes is shown. (C) Depiction of the boxed area in (B) illustrating the vectors used to calculate the distance, d , between e1 and the centroid of nucleus 3 when projected onto the line segment extending between myotube ends. (D) Illustration of the myotubes following projection of all nuclei onto the line segment between e1 and e2. Myotubes are aligned at the midline of the cells. (E) Example of a nuclear distribution plot for a population of untreated myotubes. Each line on the y-axis represents an individual myotube, organized according to length. The ends of the myotube are marked with a dark square; data points represent individual nuclei.