Imaging the effector CD8 synapse

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Summary

Here we describe 4D imaging of effector CD8+ T cells as they conjugate and kill live targets *in vitro* and analyse the polarisation dynamics of intracellular compartments to this cell-cell interface.

Running head: Imaging CD8 synapses

Keywords

Nucleofection, timelapse confocal imaging, Imaris, 4D Object-based image analysis.

1. Introduction

The immune synapse of effector CD8+ T cells or cytotoxic T lymphocytes (CTL) is crucial to the eradication of intracellular pathogens and tumour cells *(1-3)*. With the advent of genetic techniques and high-speed confocal microscopy it is now relatively straightforward to mark proteins with fluorescent tags and investigate their 3D localisation during synapse formation *(4-6)*. Unlike fixed population based approaches, individual CTL-target interactions may be followed in real time, allowing the dissection of key stages in the killing process from initial interaction all the way through to detachment of the CTL and death of the target *(7)*. Whilst this provides an excellent overall picture of a protein or organelle's spatiotemporal dynamics, in as near to an *in vivo* way as possible, it does have serious limitations. First, the expression of exogenous DNA is often a problem with immune cells and correct localisation, as well as the effects of overexpression on cell behaviour, must always be verified. Second, the process is slow. To capture a CTL target interaction one relies on luck and skill to find an appropriately fluorescent CTL about to attack, that the CTL attacks during the film whilst remaining in frame and that it does not interact with other CTLs or targets during the attack. Data capture may then be followed by processor intensive rendering and analysis to quantify the depth of information each interaction contains, before finding some way to represent this in a meaningful manner. It should also be noted that conjugated cells rarely sit still and that the synapse itself is a dynamic interface, as such it rarely lies directly in one plane and often aligns to the less-well resolved z-plane.

We describe here our technique for time-lapse imaging of the transgenic OT-I *(8)* CTL system attacking the thymoma target EL-4 loaded with the OVA antigenic peptide. This technique may be easily altered to allow for other systems such as anti-allogeneic stimulated CTL or alternative targets with better markers of cell death. Such alterations are highlighted in the notes.

2. Materials

Equipment:

1. Amaxa nucleofector. We use mouse T cell transfection kits with the nucleofector from Amaxa and obtain transfection efficiencies between 40% and 60%, varying between DNA constructs. Target cells are stably transformed with membrane marker proteins using retroviral vectors.
2. Spinning disk confocal microscope. We use an inverted microscope[1] with an Olympus 60x silicon oil objective lens, incubator chamber[2], spinning disk[3] and iXon Ultra 888 camera[4]. (Figure 1)

3. Image analysis computer – the example object based image analysis described below requires a licence for BitPlane Imaris and its associated cell module (see **Note 1**).

Consumables:

1. 35-mm No.1.5 glass-bottom culture dishes with 14mm inset (MatTek)
2. Amaxa nucleofection kit for mouse T cells (human T cell kits are also available)

Media:

All media should be stored at 4°C, preferably in the dark. Components marked with * should be kept at 4°C whilst ** should be stored in aliquots at -20°C and thawed before mixing.

1. CTL medium (CTLM): Roswell Park Memorial Institute (RPMI) 1640 medium* supplemented with 10% foetal bovine serum (FBS)**, 1mM L-glutamine**, 1mM sodium pyruvate*, 100 units/ml penicillin with 0.1mg/ml streptomycin*, 50 μ M 2-mercaptoethanol** and recombinant murine Interleukin 2** (Peprotech). Please note, this medium has a lifespan of 2 weeks from preparation.

2. Target medium (TM): Dulbecco's modified eagle medium* (DMEM) supplemented with end concentrations of 10% FBS**, 1mM L-glutamine** and 100 units/ml penicillin with 0.1mg/ml streptomycin*
3. Serum-free (SFM): DMEM*
4. Imaging buffer (IB): Phenol red free RPMI 1640* with 10%FBS**, 1mM L-glutamine**, 1mM sodium pyruvate (GIBCO)*, 25mM HEPES

Cells (see **Note 2**):

1. Fluorescent target cells at ~exponential growth stage (see **Note 3**) - $2x10^5$ cells per dish
2. Activated $CD8^+$ CTL from day 5-8 post activation - $5x10^6$ per 3 dishes (roughly 2h of imaging)

Target presentation components

$OVA_{257-264}$ SIINFEKL peptide or anti-CD3 antibody (we use hamster anti-mouse clone 145-2c11 or mouse anti-human clone UCHT1 (RUO) both from BD Pharmingen)

Components for coating dishes

1. Intercellular adhesion molecule (ICAM)-1 (R&D Systems)
2. Dulbecco's phosphate buffered saline (PBS)

3. Methods

Preparation one day before imaging

1. Aliquot IB (~12ml/dish) and SFM (~15ml/ batch of 2-3 dishes) into T-25 flasks and leave to equilibrate in an incubator at 37°C 8%CO_2 overnight.
2. Coat 35-mm glass-bottom culture dishes with 1 μ g/ml murine ICAM-1 by applying 250 μ l/dish of 1 μ g/ml ICAM-1 in PBS and leaving overnight at 4°C (see **Note 4**).
3. Ensure there will be enough healthy target cells for the following day.
4. Nucleofect CTL 24 h in advance of microscopy following the instructions for the Amaxa nucleofection kit. The details for the murine CTL kit are outlined in brief below (see **Note 5**).
    a. Add 10 μ l nucleofection medium component B to 1 ml nucleofection medium in a 12-well plate and warm at 37°C, 8% CO_2 for 30 minutes.
    b. Take $5x10^6$ CTL and wash 2x with PBS.

c. Add 2.5-10 μ g total DNA constructs in <10 μ l volume to a sterile 1.5 ml Eppendorf tube (see **Note 6**).
d. Resuspend CTL in 100 μ l nucleofection solution, apply to the DNA, mix and transfer to a nucleofection cuvette (see **Note 7**).
e. Nucleofect with program X-001 mouse $CD8^+$ T cell (see **Note 8**).
f. Immediately transfer to the pre-warmed medium (5a) in the 12-well plate.
g. Return to the incubator for 2-4 h.
h. Top up to 3 ml with pre-warmed CTLM, spread evenly amongst 6 wells of a 12-well plate and then top these up to 3.5 ml with pre-warmed CTLM and return to the incubator until used for imaging.

Day of imaging

5. Start up the microscope including appropriate heating and CO_2 chambers and ensure it is ready and functional for the day.
6. Pre-warm the ICAM-1 coated glass bottom dishes in the incubator at 37°C 8% CO_2.
7. Take 5 ml of targets and centrifuge for 5 minutes at 1200 rpm in Beckman Coulter SX4400 rotor (277xg).
8. Resuspend in 1ml TM with 1 μ M SIINFEKL peptide (see **Note 9**).
9. Incubate at 37°C for 1 hour, with gentle resuspension every 15 minutes.
10. Add 9 ml prewarmed TM or SFM (see **Note 10**) and take sample for counting.
11. Centrifuge 5 minutes 1200rpm (SX4400 rotor) (277xg)
12. Whilst centrifuging:
    a. Count and calculate the number of target cells in the tube and therefore the volume for resuspension at $0.7x10^6$ cells /ml (see **Note 11**).
    b. Gently wash unbound ICAM-1 off of the preheated imaging dishes with PBS 3 times
13. Resuspend targets at $0.7x10^6$/ml in SFM or TM (see **Note 10**) and apply 250 μ l to each dish.
14. Transfer to incubator for 5 minutes (see **Note 12**).
15. Gently rinse off unbound targets by application of 1.5 ml IB to the dish (see **Note 13**).
16. Return dishes to the incubator until ready for use on the microscope.
17. Take ~10^6 nucleofected CTL (1-2 wells from 12-well plate) into a total volume of 10 ml IB and centrifuge at 1000 rpm (SX4400 rotor) (193xg) for 5 min.

18. During centrifugation of CTL ensure dish is correctly set up on the microscope and the settings are ready for imaging (see **Note 14**).
19. Pour off the supernatant from the CTL keeping ~150 μ l on the pellet
20. Pipette the cells in this volume up and down, whilst keeping warmly cupped in the hand and apply to the dish on the microscope in a gentle drop wise manner (see **Note 15**).
21. Search for appropriate CTL:target interactions and begin imaging.
22. Repeat steps 17-21 until dishes with targets are used, then repeat from 6-21 until all ICAM-1 coated dishes are used.
23. Export the data and visualise/analyse with software such as Imaris (see **Notes 16 & 17**).

(Figure 2)

<u>Analysis using Imaris</u>

24. Convert microscopy data to the Imaris file-type and save to a local drive (see **Note 16**).
25. Crop to the cell of interest (see **Note 16**).
26. Generate 'surfaces' of the CTL and target cell. See figure 3.

(Figure 3)

27. Use the target cell 'surface' to generate a mask of the target cell channel.

(Figure 4)

28. Use the CTL surface to mask this newly generated surface. See figure 5.

(Figure 5)

29. Use the 'cell' module to search this new channel for 'nuclei'.

(Figure 6)

30. Detect organelles/ intracellular compartments as 'spots'.

(Figure 7)

31. Complete the cell module with or without tracking.

(Figure 8)

32. Export statistics. Use 'closest nucleus distance' for IS polarisation dynamics.

33. Repeat for next cell.

4. Notes

1. Programs such as Imaris require a lot of computer processing power for 4D data sets. See: http://www.bitplane.com/systemrequirements.aspx. Generally the local drive speed controls how long it takes to load a file and takes over from the random-access memory (RAM) when it is filled, the RAM tries to hold the data for calculation and the graphics card controls data representation, whilst the processor does the modelling calculations.

2. Any CTL model may be used here providing both CTL and target are fluorescent by the day of imaging.
3. Many target cell lines are susceptible to nucleofection (Methods 4) and may be repeatedly sorted for stable expression. Alternatively dyes such as PKH26 and CFSE may be successfully employed to colour target cells before imaging *(9)*. Common cell lines used in our lab are EL4 which carry the H2-K^b MHC for the OT-I system and P815 for both anti-H2-D^d allogeneic CTL and redirected lysis where the Fc receptor (FcR) expression on these cells allows capture of anti-CD3 antibody for CTL activation.
4. The concentration of ICAM-1 we use is optimised to allow the CTL to move across the dish without being activated or adhere too tightly.
5. Other CTL nucleofection systems predominately vary in the naming of the media and solutions but follow the same basic protocol. Alternatively CTL lines stably expressing fluorescent markers of interest may be used.
6. It is important to consider fluorescent construct combinations carefully. Modelling works best when each structure of interest can be captured in a separate channel. The analysis below uses points of high signal intensity as seed points from which to grow the cell model, so any overlap or bleed-through may result in the target or CTL models merging with one another preventing discrimination.
7. Remove as much supernatant as possible before resuspension. Remaining PBS can lead to sparking during the nucleofection process. We find the nucleofection solution can be harmful to cells with time. It is therefore advisable to keep CTL in this for as short a time as possible and we often split the process into batches of 4 before resuspending the next set of CTL in the nucleofection solution.

8. Or whichever is appropriate for the system used.
9. For redirected lysis systems where it is necessary to stimulate conjugate formation with anti-CD3 antibody presentation, replace peptide loading (steps 8&9) instead with antibody at 0.5 μ g/ml at step 8 and step 9 may be shortened to around 15 minutes. Please note this requires FcR expressing target cells such as P815.
10. Some target cells adhere to the coverslip readily in IB, while others (such as EL4) adhere better when plated in SFM.
11. If larger targets are being used it may be necessary to reduce the concentration to achieve isolated target cells.
12. The length of time required for cells to adhere to the dish will vary and optimal timing needs to be established for each cell type.
13. This is easiest done by gently tilting the dish and applying the IB close to the edge of the dish above the centre (see below).

(Figure 9)

14. Beware the time it takes for some objective lens oil to adjust to 37°C. If the oil temperature is not equilibrated to the microscope the change in temperature may alter the focal height across the imaging session.
15. CTL have a tendency to clump and it is essential to separate them to ensure what is imaged is not a co-attack of a non-fluorescent 'ghost' cell.
16. The data generated from this time-lapse 3D imaging can be considerably larger than most fixed or 2D experiments, with individual data files reaching 20-40Gb. This poses considerable strain on data transfer and storage systems as well as later analysis where linear file size increases can lead to exponential growths in processing time. It is therefore highly recommended to optimise all computer systems where possible and pre-process data appropriately before more intense analysis.
17. There are many free packages available that may be forced to do similar analysis to Imaris, but this is a very intuitive program for navigating 4D data and its modelling functions allow user input to manually distinguish objects where algorithms alone cannot.

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Figure Legends

Figure 1. Confocal spinning disk microscope. 1. Inverted microscope with an Olympus 60X silicon oil objective lens. 2. Electronic stage with incubator chamber. 3. Yokogawa spinning disk. 4. iXon Ultra 888 camera.

Figure 2. Example time-lapse data. Maximum intensity projection of EL4 expressing $mTagBFP_2$ (blue), presenting OVA to an OT-I CTL nucleofected with Lifeact-EGFP (binds f-actin *(10)*) (green), microtubule end-binding protein 3 (EB3)-iRFP670 *(11)* (white) and expressing Gzm-B-TdTomato (granzyme B, protease found in cytolytic granules *(12)*) (red) from an endogenous promoter.

Figure 3. Modeling the CTL and target as surfaces. A, Preview of target surface at the threshold value selection stage. This should be chosen so as to generate a solid surface at the synapse without excessive dilation into the CTL, as this will define the surface to which distances will be calculated. B, Finished target surface. C, CTL surface preview at the threshold value selection stage. As with the target threshold, aim for a solid synapse without expanding too far into the target. D, CTL surface generated. E, Both target and CTL surfaces generated.

Figure 4. Masking the target cell. A, Start of masking process to leave just the target volume with values >0. B, Masked target cell channel generated.

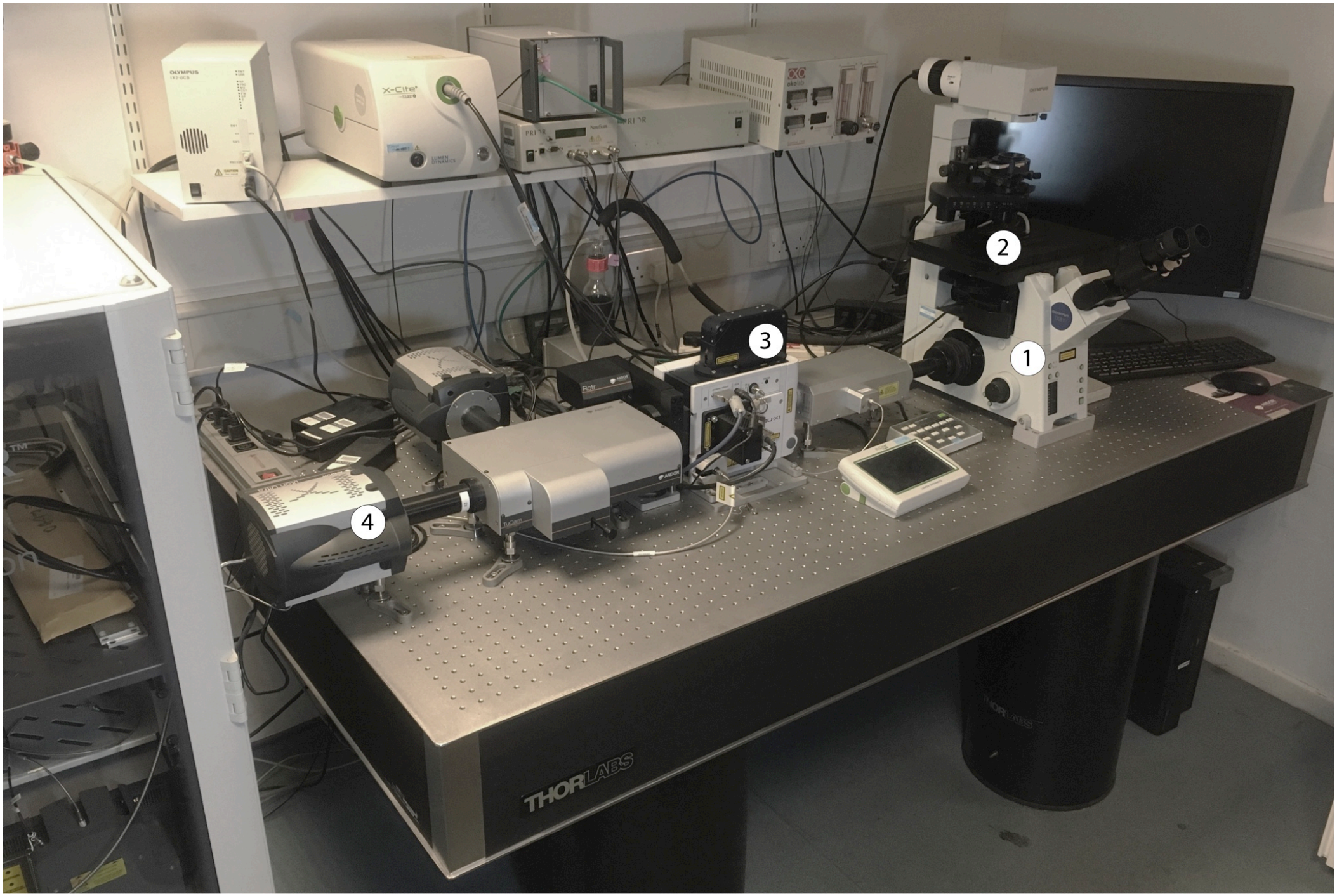
Figure 5. Generating the 'synapse' channel. A, start of the masking process to set all pixels not at the CTL-target boundary to 0. B, The generated synapse channel in blue.

Figure 6. Defining the synapse channel as the 'nucleus' of the cell module.

Figure 7. Defining the intracellular structures of interest. The algorithm is based on the 'spots' algorithm and locates approximate spheres of intensity within a chosen channel. The EB3-iRFP670 channel has been chosen here to demonstrate that weak signals may be modelled when the background is low.

Figure 8. The completed cell model.

Figure 9. Gently washing excess cells off of the glass.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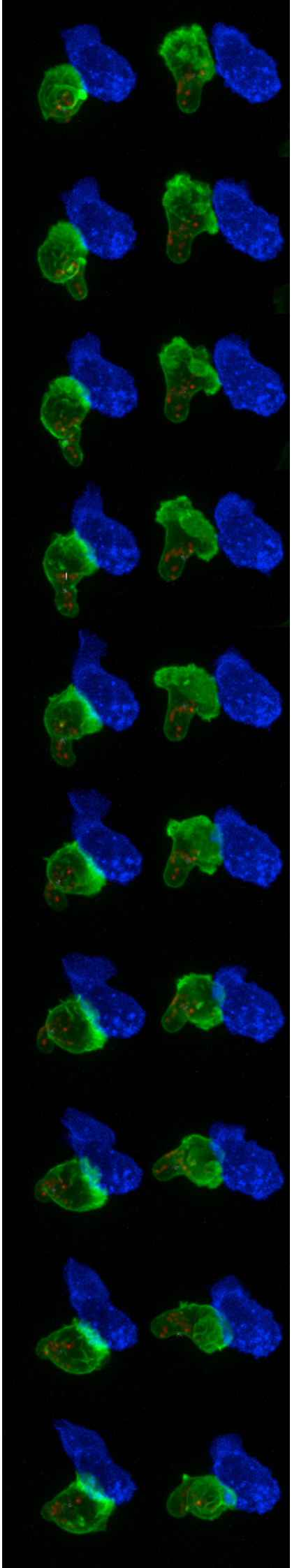

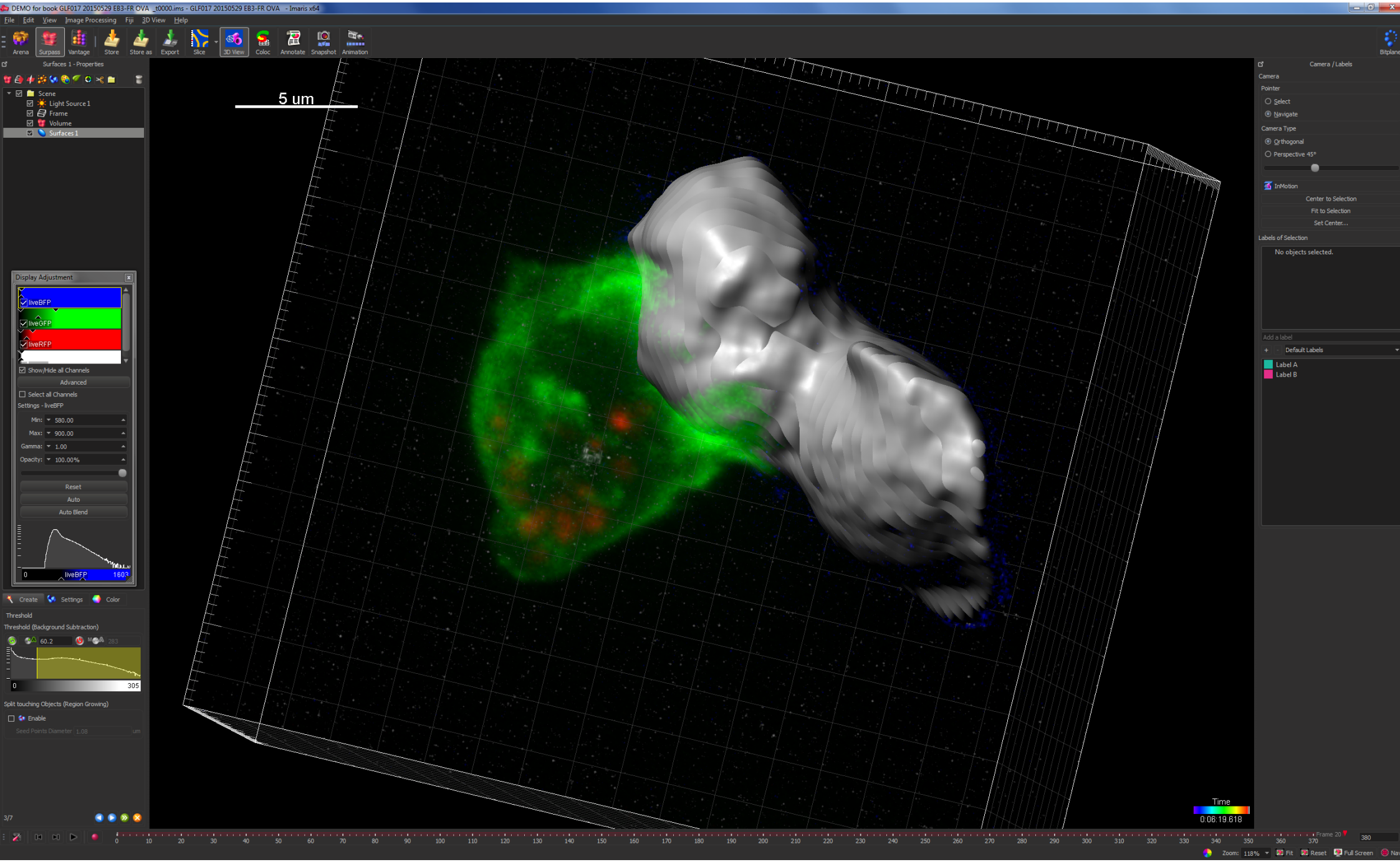
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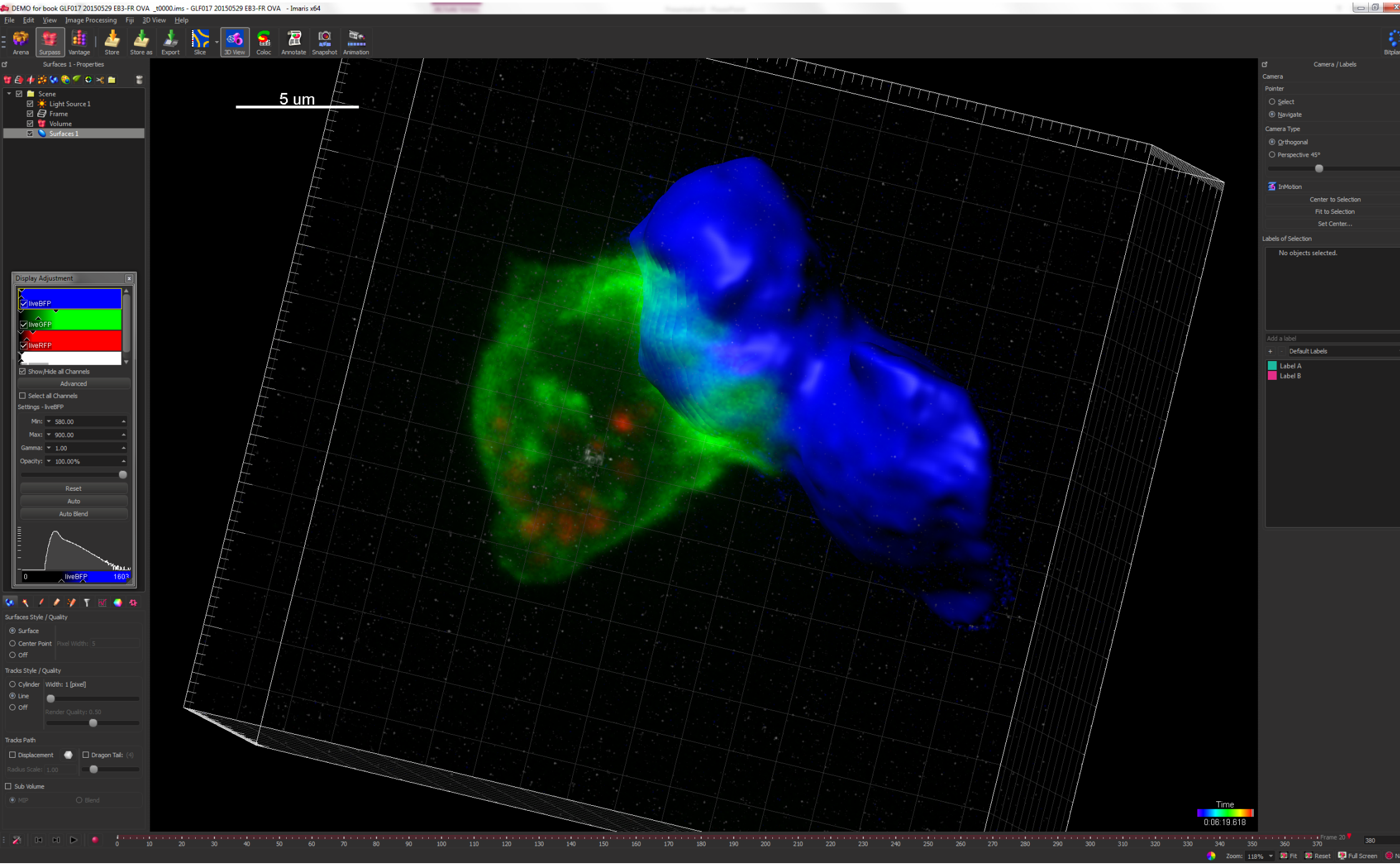
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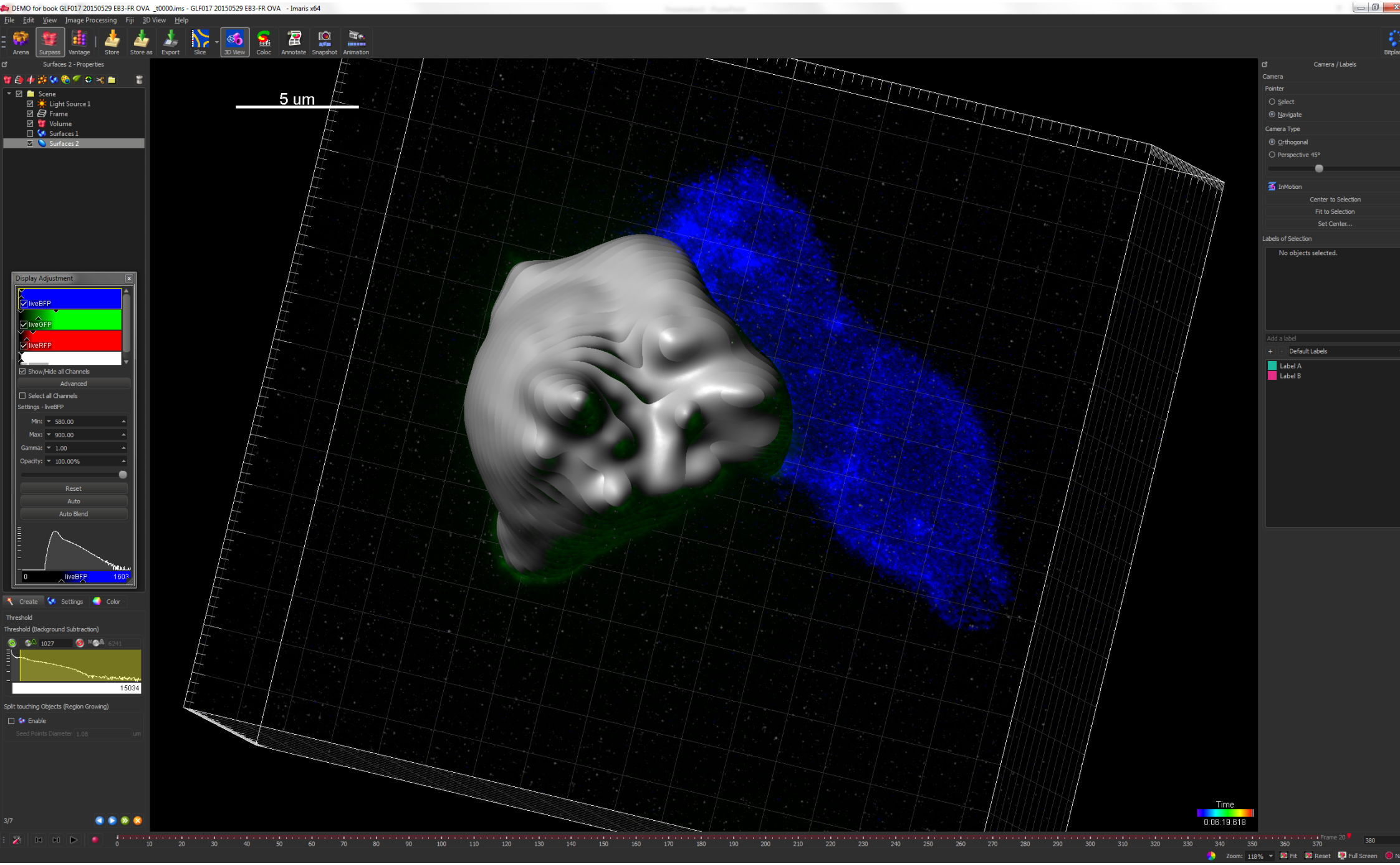
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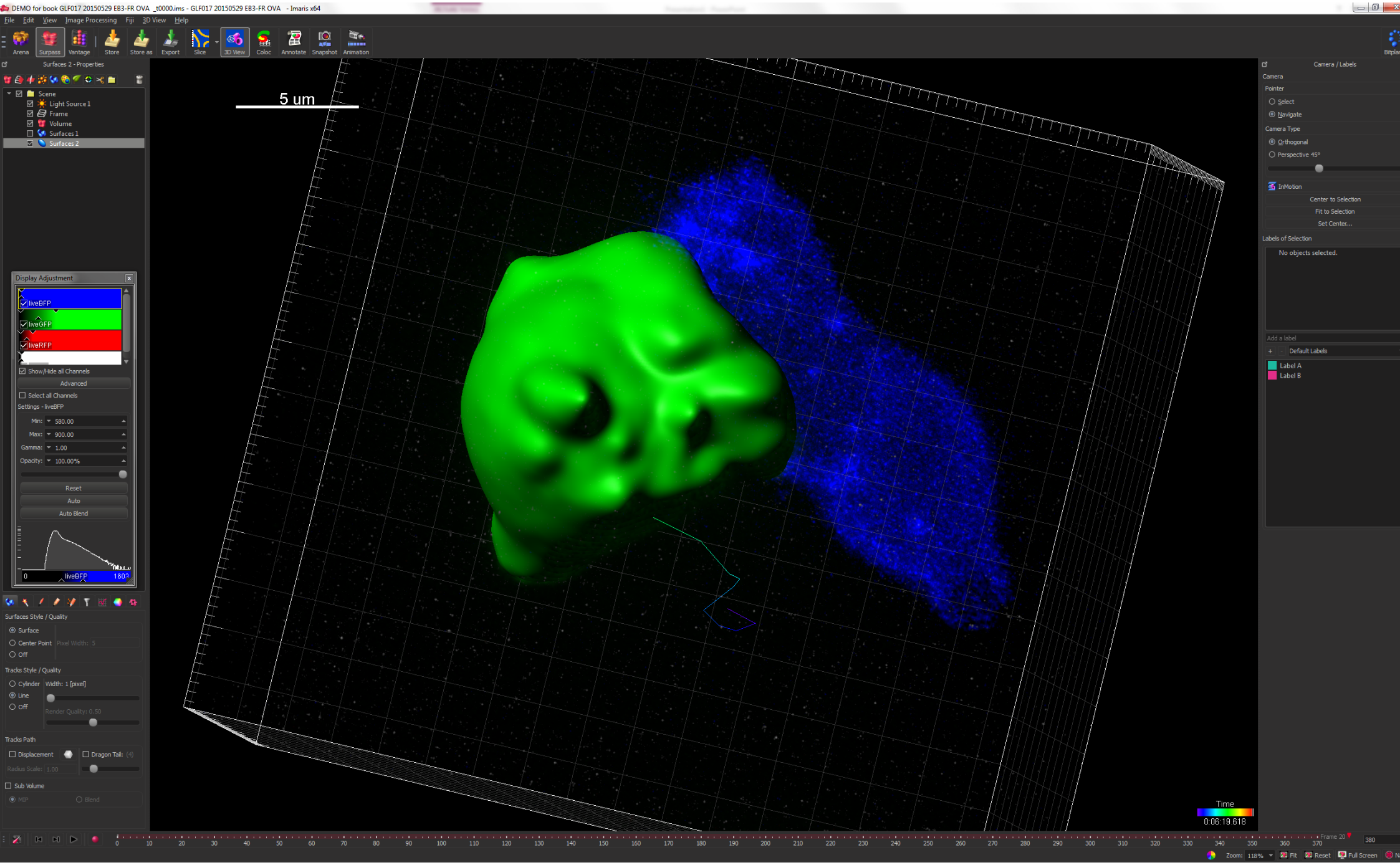
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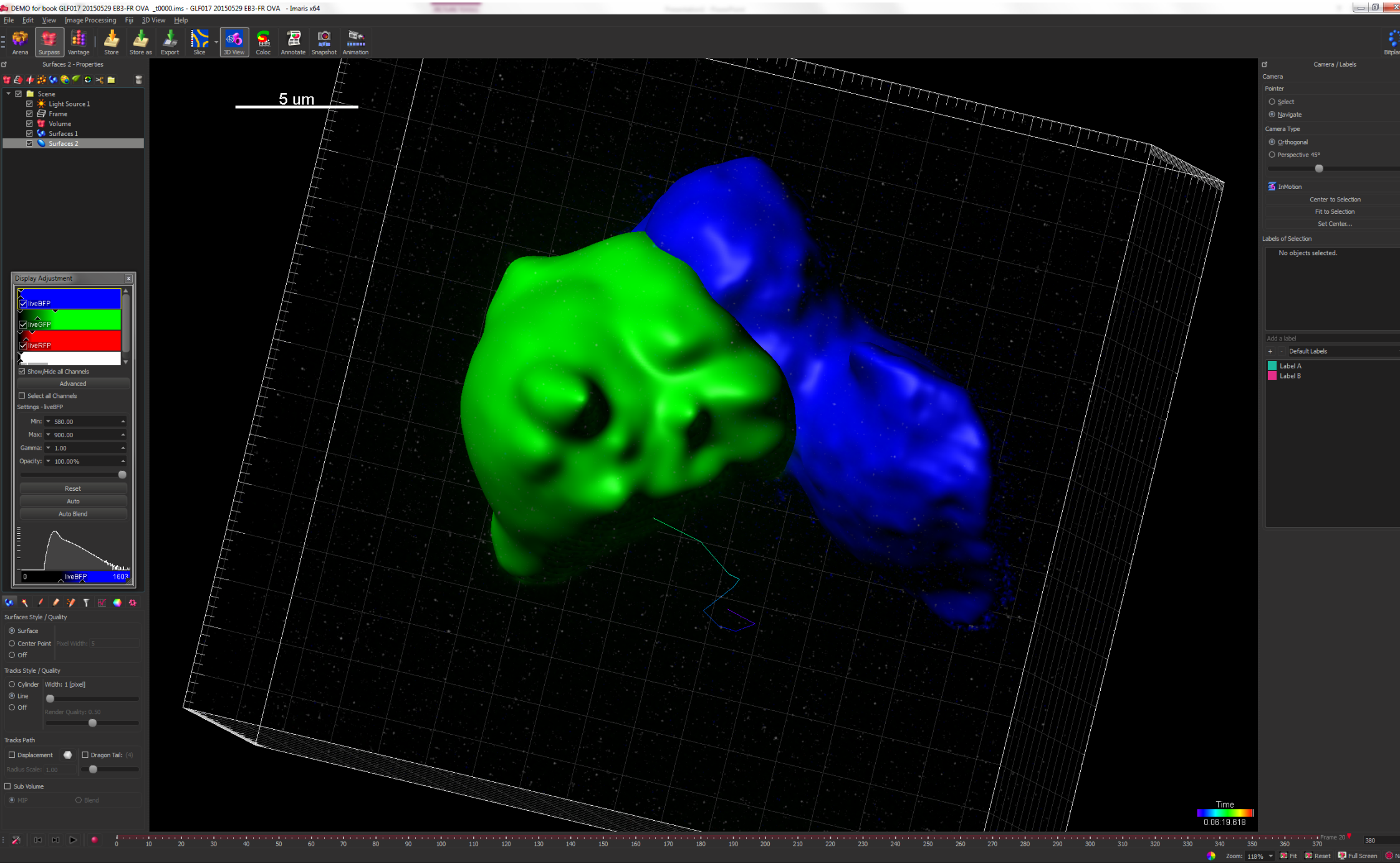
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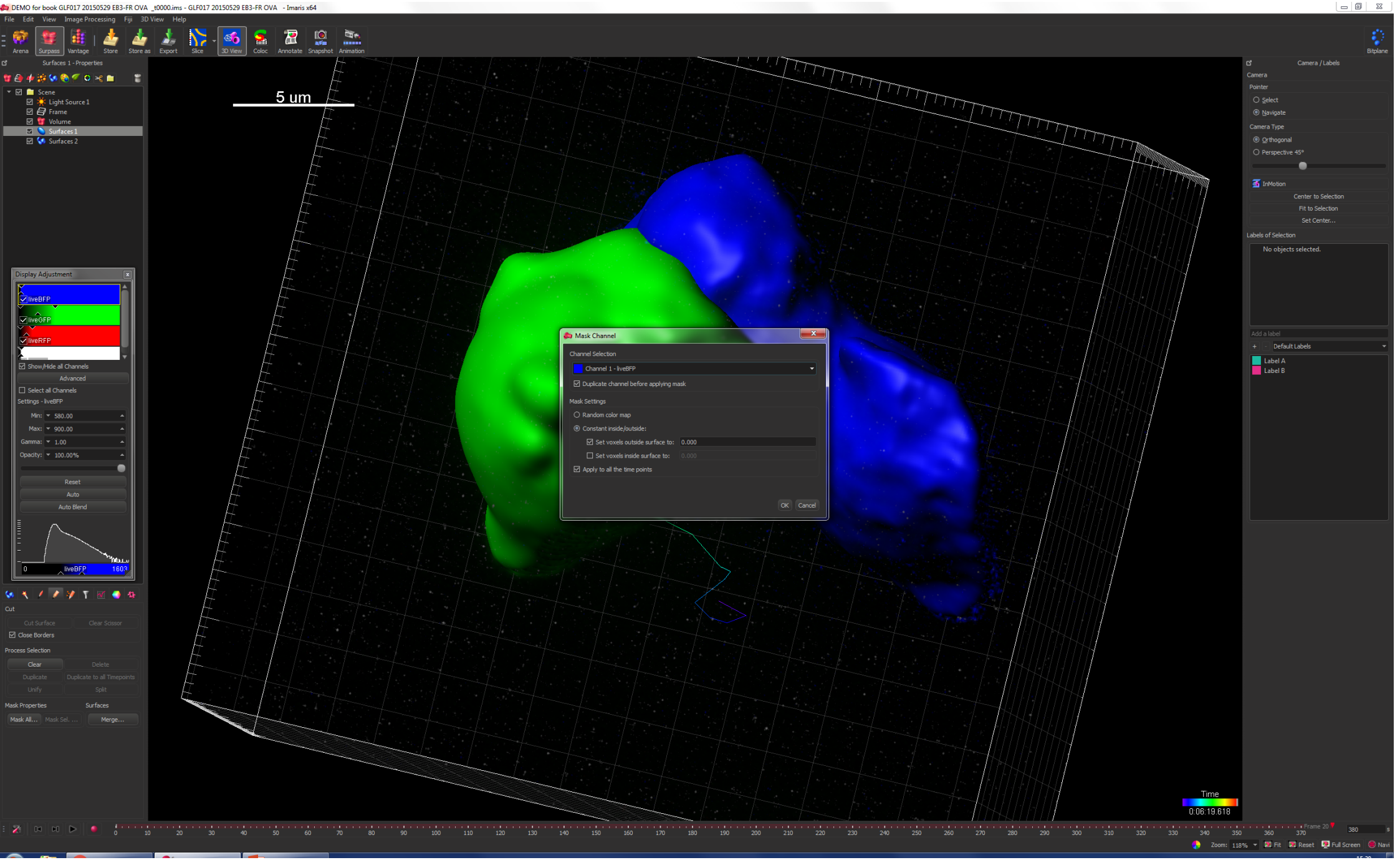
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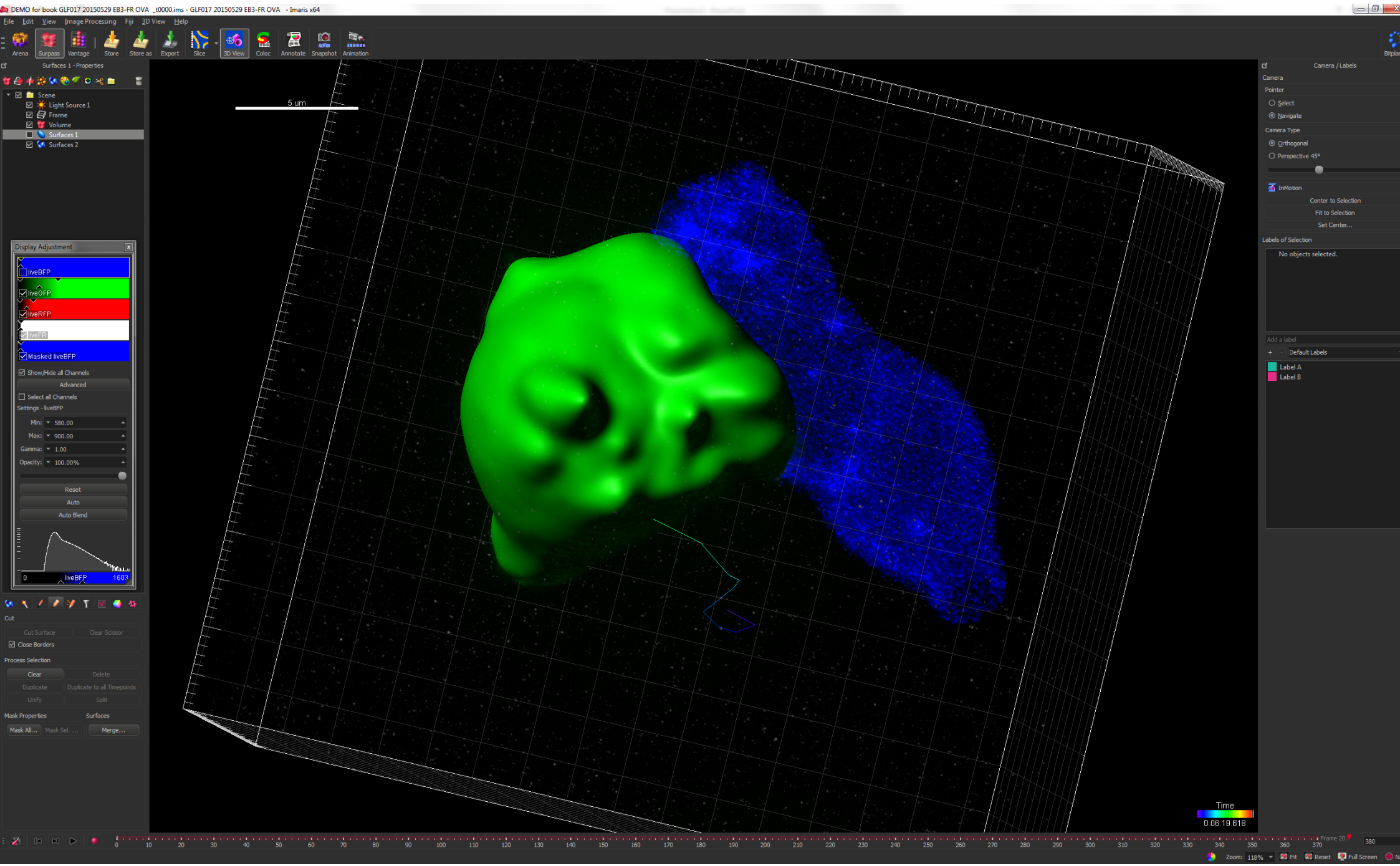
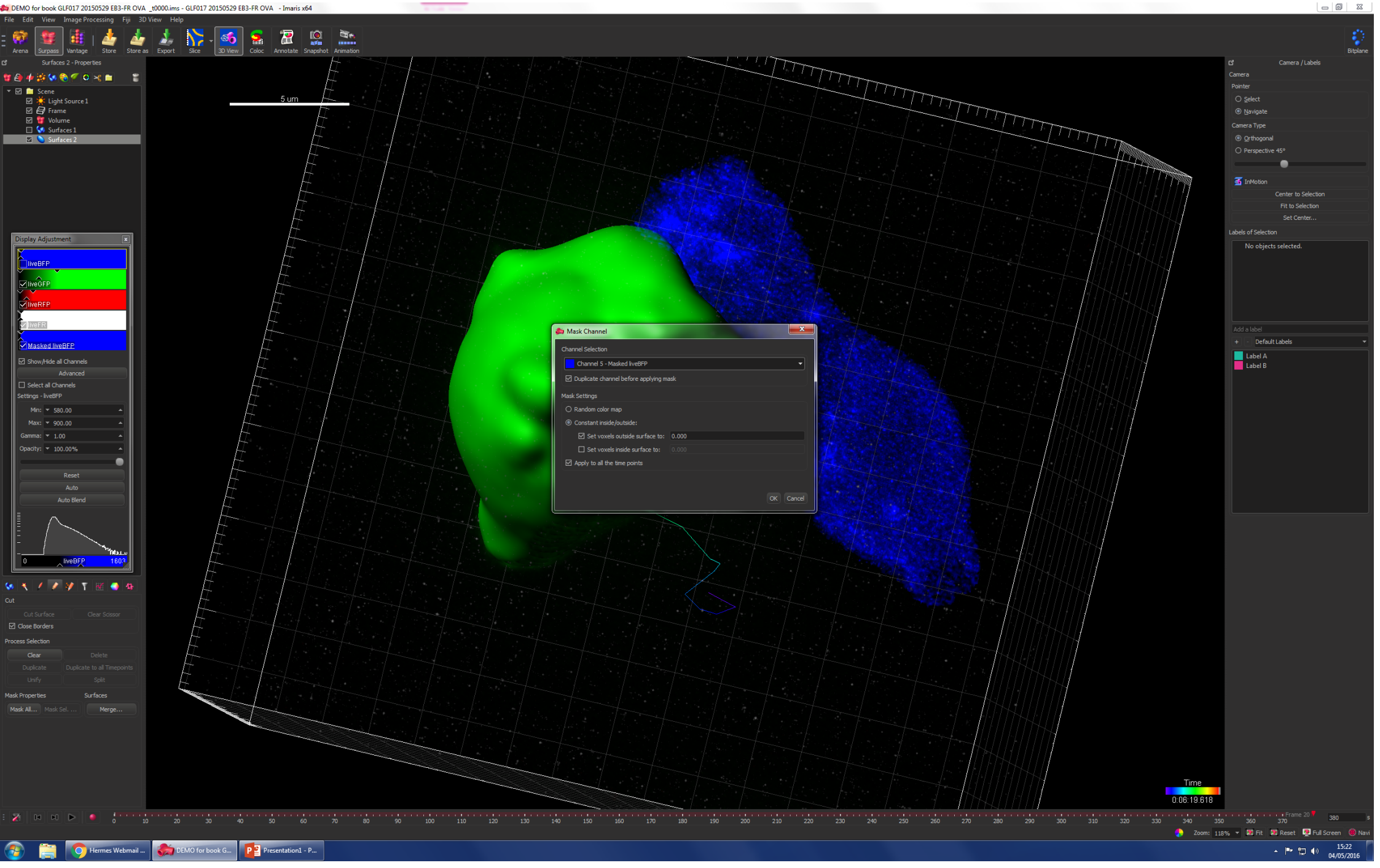
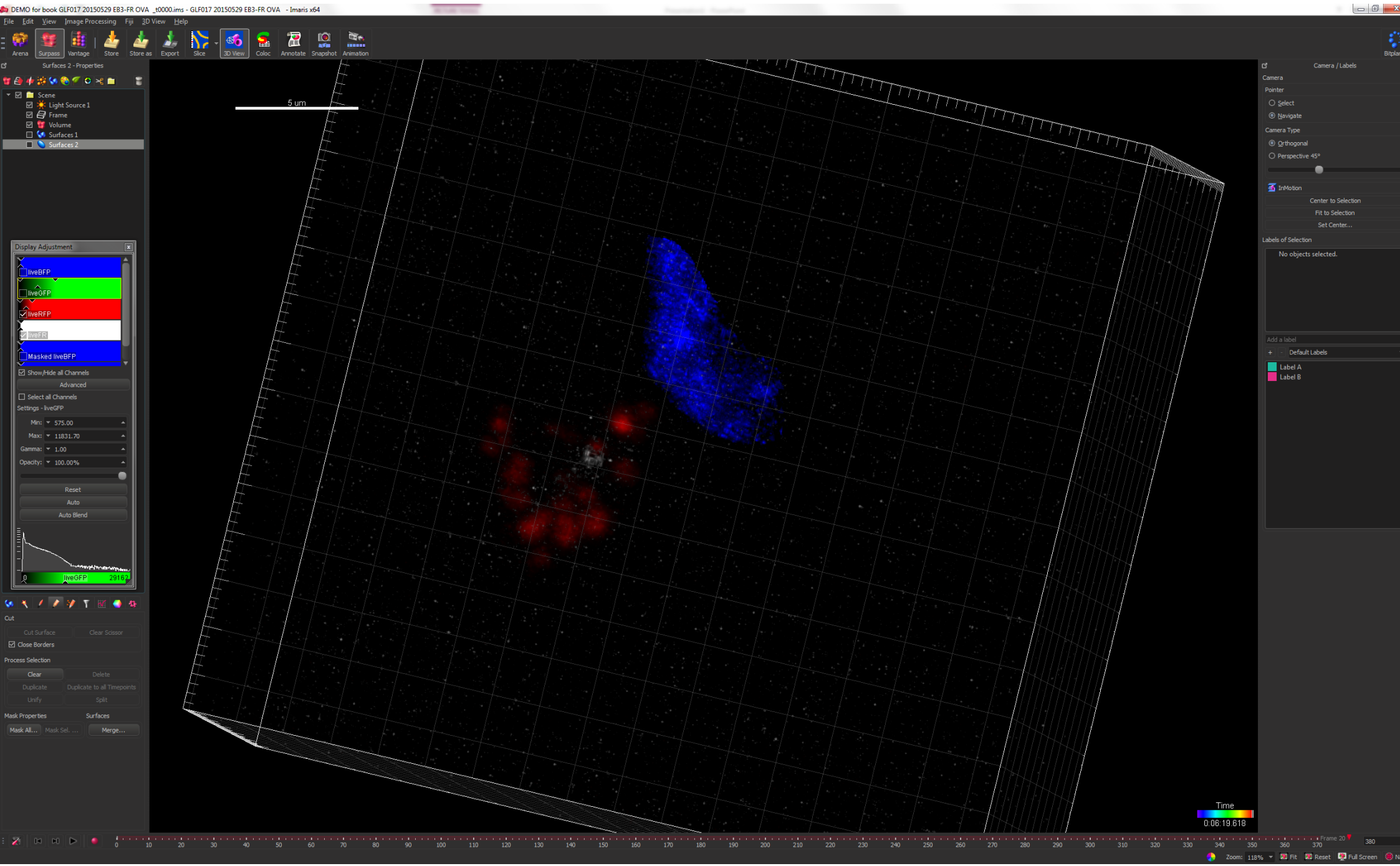
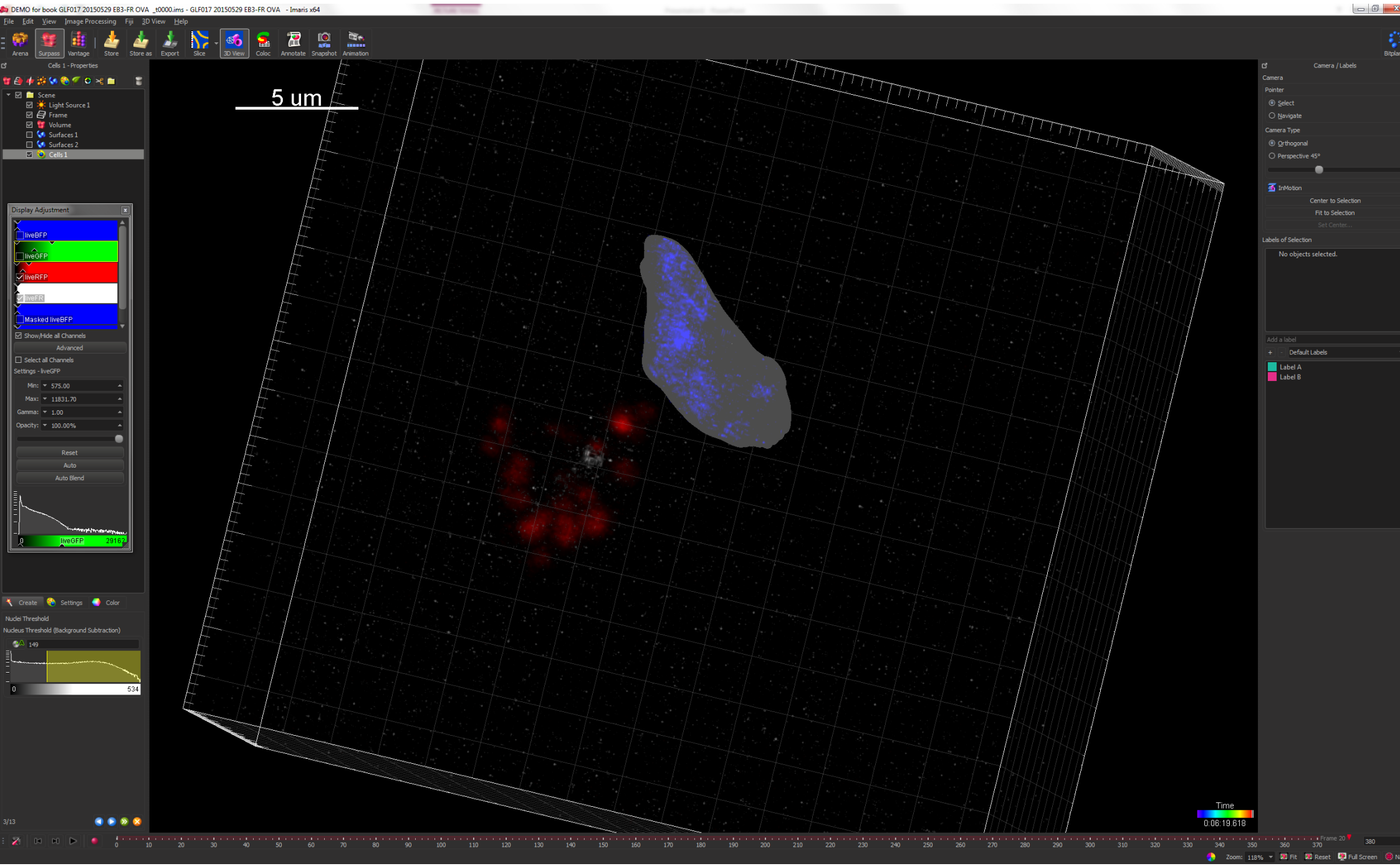
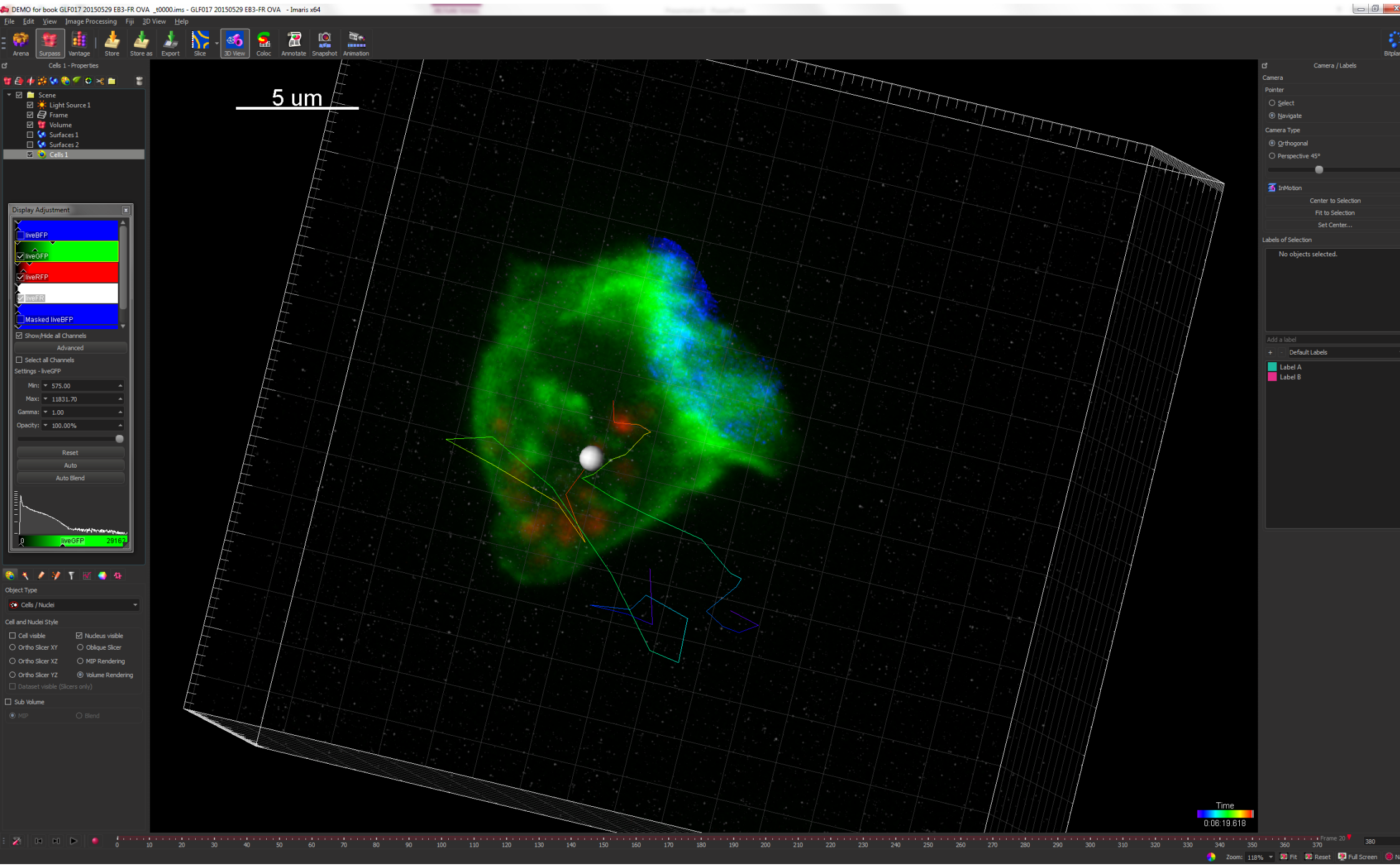
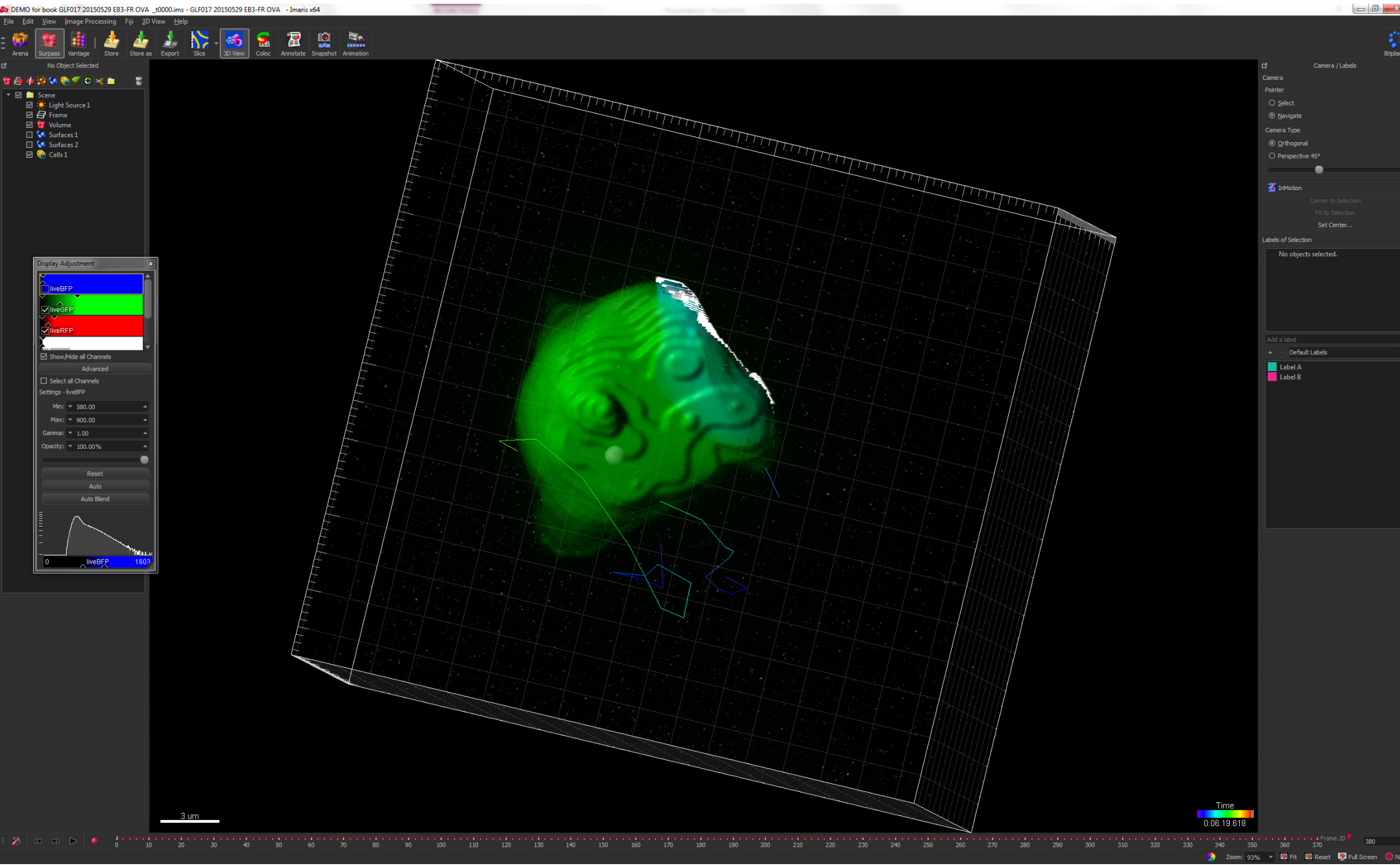

DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation
Surfaces 1 - Properties
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
Display Adjustment
liveBFP
liveGFP
liveRFP
liveFR
Masked liveBFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveBFP
Min: 580.00
Max: 900.00
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
0
liveBFP
160³
Cut
Cut Surface
Clear Scissor
Close Borders
Process Selection
Clear
Delete
Duplicate
Duplicate to all Timepoints
Unify
Split
Mask Properties
Surfaces
Mask All...
Mask Sel. ...
Merge...
5 um
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
Time
0:06:19.618
Zoom: 118%
Fit
Reset
Full Screen

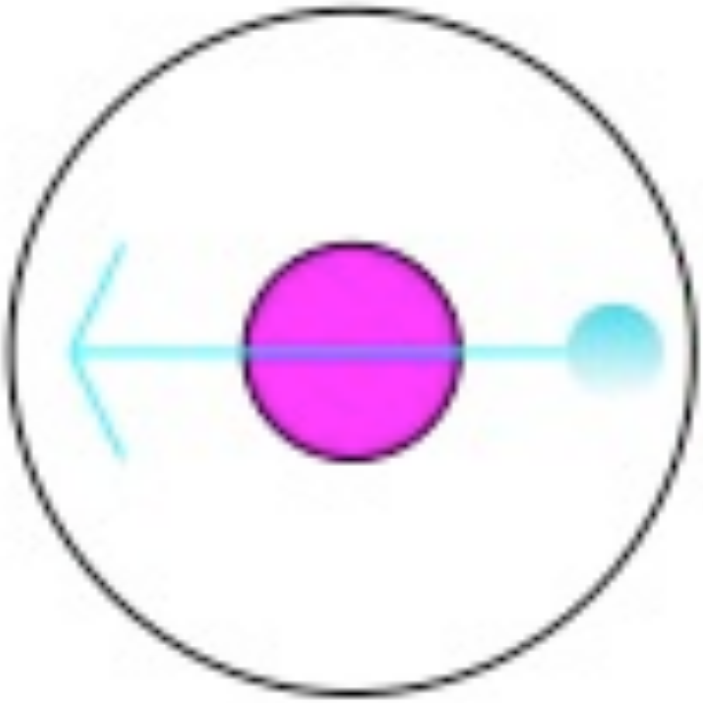

DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation Bitplane
Surfaces 2 - Properties
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
5 um
Display Adjustment
liveBFP
liveGFP
liveRFP
liveFR
Masked liveBFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveBFP
Min: 580.00
Max: 900.00
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
Cut
Cut Surface
Clear Scissor
Close Borders
Process Selection
Clear
Delete
Duplicate
Duplicate to all Timepoints
Unify
Split
Mask Properties
Surfaces
Mask All...
Mask Sel. ...
Merge...
Mask Channel
Channel Selection
Channel 5 - Masked liveBFP
Duplicate channel before applying mask
Mask Settings
Random color map
Constant inside/outside:
Set voxels outside surface to: 0.000
Set voxels inside surface to: 0.000
Apply to all the time points
OK
Cancel
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
Time
0:06:19.618
Zoom: 118%
Fit
Reset
Full Screen
Navi
Hermes Webmail ...
DEMO for book G...
Presentation1 - P...
15:22
04/05/2016

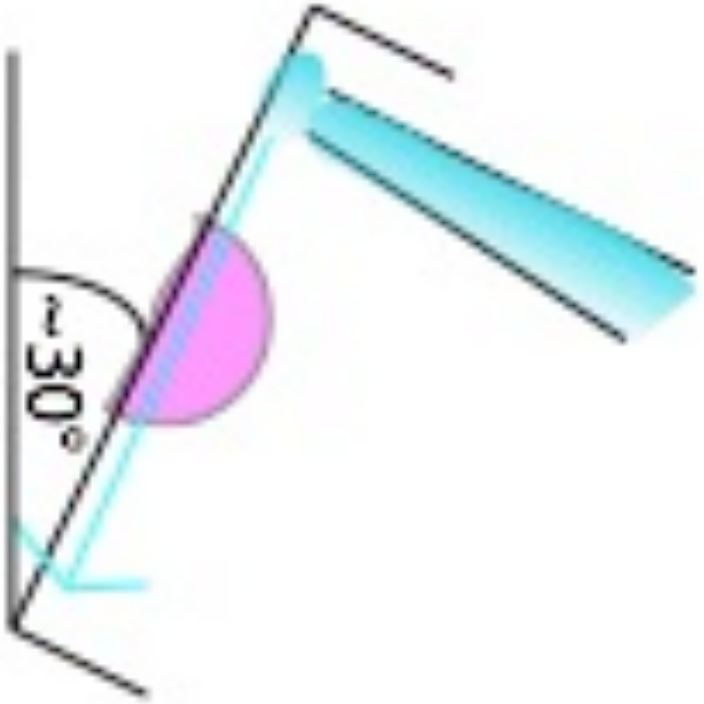

DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation
Surfaces 2 - Properties
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
Display Adjustment
liveBFP
liveGFP
liveRFP
liveFR
Masked liveBFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveGFP
Min: 575.00
Max: 11831.70
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
0
liveGFP
29162
Cut
Cut Surface
Clear Scissor
Close Borders
Process Selection
Clear
Delete
Duplicate
Duplicate to all Timepoints
Unify
Split
Mask Properties
Surfaces
Mask All...
Mask Sel. ...
Merge...
5 um
Time
0:06:19.618
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
Zoom: 118%
Fit
Reset
Full Screen


DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation
Cells 1 - Properties
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
Cells 1
Display Adjustment
liveBFP
liveGFP
liveRFP
liveFR
Masked liveBFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveGFP
Min: 575.00
Max: 11831.70
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
0
liveGFP
29167
Create Settings Color
Nuclei Threshold
Nucleus Threshold (Background Subtraction)
149
0
534
3/13
5 um
Time
0:06:19.618
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
Zoom: 118%
Fit
Reset
Full Screen


DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation
Cells 1 - Properties
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
Cells 1
Display Adjustment
liveBFP
liveGFP
liveRFP
liveFR
Masked liveBFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveGFP
Min: 575.00
Max: 11831.70
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
Object Type
Cells / Nuclei
Cell and Nuclei Style
Cell visible
Nucleus visible
Ortho Slicer XY
Oblique Slicer
Ortho Slicer XZ
MIP Rendering
Ortho Slicer YZ
Volume Rendering
Dataset visible (Slicers only)
Sub Volume
MIP
Blend
5 um
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
Time
0:06:19.618
Zoom: 118%
Fit
Reset
Full Screen


DEMO for book GLF017 20150529 EB3-FR OVA _t0000.ims - GLF017 20150529 EB3-FR OVA - Imaris x64
File Edit View Image Processing Fiji 3D View Help
Arena Surpass Vantage Store Store as Export Slice 3D View Coloc Annotate Snapshot Animation
No Object Selected
Scene
Light Source 1
Frame
Volume
Surfaces 1
Surfaces 2
Cells 1
Display Adjustment
liveBFP
liveGFP
liveRFP
Show/Hide all Channels
Advanced
Select all Channels
Settings - liveBFP
Min: 580.00
Max: 900.00
Gamma: 1.00
Opacity: 100.00%
Reset
Auto
Auto Blend
0 liveBFP 1603
Camera / Labels
Camera
Pointer
Select
Navigate
Camera Type
Orthogonal
Perspective 45°
InMotion
Center to Selection
Fit to Selection
Set Center...
Labels of Selection
No objects selected.
Add a label
Default Labels
Label A
Label B
3 um
Time
0:06:19.618
Zoom: 93%
Fit
Reset
Full Screen




~30°