STAR Protocols

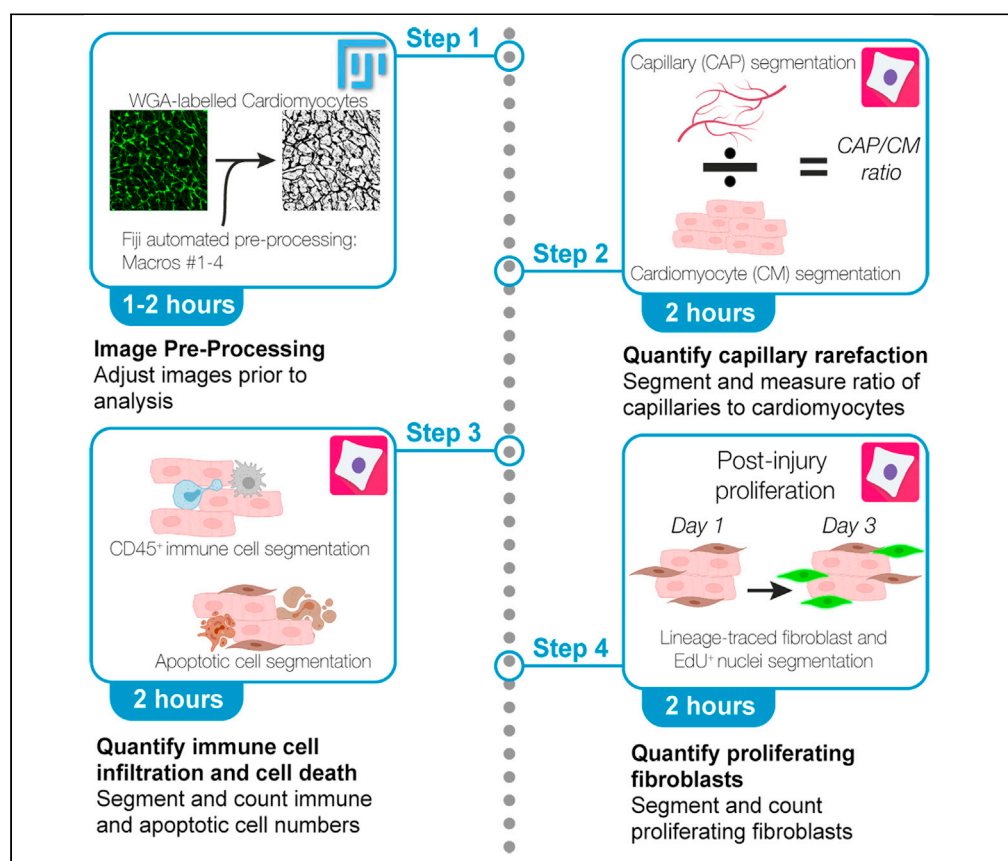**Protocol**

An image analysis protocol using CellProfiler for automated quantification of post-ischemic cardiac parameters

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Highlights

Automated pipelines
for high-throughput
analysis of cardiac
imaging data

Focus on image
processing and
segmentation to
derive common
functional cardiac
measures

Scalable for use in
other tissue types

Quantitative assessment of post-ischemic cardiac remodeling is often hampered by tissue
complexity and structural heterogeneity of the scar. Automated quantification of microscopy
images offers an unbiased approach to reduce inter-observer variability. Here, we present a
CellProfiler-based analytical pipeline for the high-throughput analysis of confocal images to
quantify post-ischemic cardiac parameters. We describe image preprocessing and the
quantification of capillary rarefaction, immune cell infiltration, cell death, and proliferating
fibroblasts. This protocol can be adapted to other tissue types.

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Protocol

An image analysis protocol using CellProfiler for automated quantification of post-ischemic cardiac parameters

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SUMMARY

Quantitative assessment of post-ischemic cardiac remodeling is often hampered by tissue complexity and structural heterogeneity of the scar. Automated quantification of microscopy images offers an unbiased approach to reduce inter-observer variability. Here, we present a CellProfiler-based analytical pipeline for the high-throughput analysis of confocal images to quantify post-ischemic cardiac parameters. We describe image preprocessing and the quantification of capillary rarefaction, immune cell infiltration, cell death, and proliferating fibroblasts. This protocol can be adapted to other tissue types.
For complete details on the use and execution of this profile, please refer to Janbandhu et al. (2021).

BEFORE YOU BEGIN

We outline an image analysis protocol for the automated analysis of post-ischemic cardiac parameters, using freely available software, Fiji (Schindelin et al., 2012) and CellProfiler (Carpenter et al., 2006; McQuin et al., 2018) (key resources table). These two open-source software are highly complementary when used in tandem for image-processing and segmentation, as well as for many other bioimage analysis needs (Dobson et al., 2021). No programming knowledge is necessary to run Fiji Macros or CellProfiler pipelines (key resources table), so this protocol is highly accessible. Theoretically, there is no limit to the numbers of images that can be analyzed using this approach, the limiting factor here is computational power. It is feasible that, using high-performance computing, tens of thousands of images could be analyzed for high throughput bioimage analysis, by calling these pipelines with the command line. There are 4 CellProfiler pipelines and image sets used here for the analysis of: 1) cardiomyocyte (CMs) hypertrophy and vascularization; 2) inflammation; 3) cardiac fibroblast (CF) proliferation and 4) apoptosis. The acquisition of high-quality 2D confocal images is necessary for accurate use of this pipeline, and is, as such, assumed. Details of tissue sectioning, staining and confocal microscopy are described in Janbandhu et al. (2021). The fluorescently conjugated markers used in the study are detailed in Table 1. CFs and their progeny were irreversibly tagged with tdTomato (TdT) using transgenic mouse models (genetic lineage-tracing), and mice were injected with 5-ethynyl-2'-deoxyuridine (EdU) 1 day before tissue collection to identify proliferating CFs. CM boundaries were labeled with Wheat Germ Agglutinin (WGA) for

Table 1. Staining markers and cardiac parameters

Staining markers	Fluorophore(s)	Label type	Cardiac parameters
TUNEL	Tetramethylrhodamine (TMR) red	Chemical reagent	Apoptosis
CD45	Cy7	Antibody	Inflammation
tdTomato and 5-ethynyl-2′-deoxyuridine (EdU)	DsRed and AlexaFluor 488	Transgenic marker and chemical reagent	CFs and proliferation
WGA (Wheat Germ Agglutinin)	AlexaFluor 488	Conjugated glycoprotein label	Hypertrophy
IB4 (Isolectin B4)	AlexaFluor 568	Conjugated lectin	Vascularization

the assessment of hypertrophy and capillaries were labeled with Isolectin B4 (IB4). Nuclei of apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TU-NEL) assay. Lastly, immune cells were labeled using a fluorescently conjugated anti-CD45 antibody.

Note: (1) If reporter mice are not available, CFs can be immuno-labeled using anti-PDGFRα antibody and visualized with appropriately labeled secondary antibody. (2) This protocol is an image analysis protocol; the availability of appropriate confocal images is assumed. The image acquisition was performed using a Zeiss LSM700 confocal microscope, and acquisition parameters used for this work were as follows: 20x Plan Apochromat air objective, with Numerical Aperture (NA) of 0.8; x/y resolution of 6.4 pixels/μm x/y size of 1024 pixels or 160.04 μm. And the timing for analysis is based on a Dual-Core Intel Core i5 processor with 8GB RAM, running macOS Catalina operation system. (3) This protocol was optimized for use with confocal images; however, it is conceivable that it could be applied to widefield fluorescent images, if they are of sufficiently high quality, with regards to signal-to-noise, intensity and tissue background. If not, then additional deconvolution steps may be necessary prior to analysis, with proprietary software such as Huygens.

△ CRITICAL: To ensure high-throughput image processing, all files should contain a unique identifier, so they can be identified after processing and analysis e.g., *Sample1_CD31_2021.lsm, Sample1_CD45_2021.lsm.*

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software		
Fiji ImageJ v1.52p	Schindelin et al. (2012) (PMID: 22743772)	https://imagej.net/Fiji
CellProfiler v3.1.9	McQuin et al. (PMID: 29969450)	https://cellprofiler.org/
Others		
Macro #1: 1_Macro_pipeline1.ijm	This paper	https://github.com/VCCRI/Cardiac-Image-Analysis.git
Macro #2: 2_ Macro_pipeline2.ijm	This paper	As above
Macro #3: 3_ Macro_pipeline3.ijm	This paper	As above
Macro #4: 4_ Macro_pipeline4.ijm	This paper	As above
Analysis pipeline #1 - Segment and measure capillaries and cardiomyocytes: 1_Capillary_per_CM_segment.cpproj	This paper	https://github.com/VCCRI/Cardiac-Image-Analysis.git
Analysis pipeline #2 - Segment and measure immune cells and cardiomyocytes: 2_CD45_per_CM_segment.cpproj	This paper	As above
Analysis pipeline - Segment and measure proliferating cells and fibroblasts: 3_Edu_FB_segment.cpproj	This paper	As above

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Analysis pipeline - Segment apoptotic cells: *4_Tunel_segment.cpproj*	This paper	As above
4 x Image folders – contains sample images (.lsm format) to run each of the 4 Macros and pipelines	This paper	https://github.com/VCCRI/Cardiac-Image-Analysis.git
Example Data folder – contains example raw .csv file outputs, segmentation outputs and annotated .xlsx file for pipeline #1 to indicate relevant data for downstream analysis	This paper	https://github.com/VCCRI/Cardiac-Image-Analysis.git

Note: The pipeline detailed below will work on the current versions of both CellProfiler (v4.2.1) and Fiji (v1.53). However, if using a later version of CellProfiler, it may be necessary to load the pipelines using the *.cpipe* file format, also found in the Github repository page, as the *.cproj* files may not load effectively. The minimum version of CellProfiler required is v3.0.

STEP-BY-STEP METHOD DETAILS
Image pre-processing

⏱ Timing: ∼25–30 s per file with Fiji

Multi-channel confocal/fluorescence microscopy images can be acquired using many different imaging platforms. Each platform will save image data and accompanying metadata with proprietary file formats, which can be read into Fiji automatically, or using the Bio-Formats importer (Linkert et al., 2010). Image pre-processing steps are essential for the accurate segmentation of objects, such as nuclei, capillaries and immune cells. Each Fiji macro (key resources table) will run batch processes on a single folder of images, to perform the following: Splitting individual channels, converting from 16-bit to 8-bit (for visualization purposes only), improving contrast and smoothing all images, and inverting WGA-stained CMs.

Note: Image pre-processing steps below are designed to be performed on the example images found in the VCCRI Github folder (link in the key resources table). Each macro will output processed images into a new folder within the user-selected directory. If using on the user's own image sets, the directory structures may be different, and the running of Macros will need to be adapted accordingly.

1. Download the VCCRI GitHub *Cardiac-Image-Analysis* repository, which contains all files listed in the key resources table.
   a. Click the green *"Code"* button on the main page.
   b. Download the zip folder and move the uncompressed directory to the desired location.
2. Open Fiji and run macro #1 (*1_Macro_pipeline1.ijm*) on image folder 1 (*1_Images_pipeline1_WGA_IB4*). This macro splits and pre-processes images for pipeline #1.
   a. Select *"Plugins > Macro > Run…"*.
   b. Select macro #1 in the first popup window, click *Open*.
   c. Select the desired input folder, and in the second popup window, click *Open*.
   d. Wait until the log states the conversion is complete, or the processing updates (in the bottom left corner of the Fiji window) have stopped, before moving to the next step.
   e. This will output a folder named "SplitChannel_1" into the selected input folder, which will be used as the input for CellProfiler pipeline #1.
3. In Fiji, run macro #2 (*2_Macro_pipeline2.ijm*) on image folder 2 (*2_Images_pipeline2_WGA_CD45*). This macro splits and pre-processes images for pipeline #2.

Fiji - Running Macros 1-4
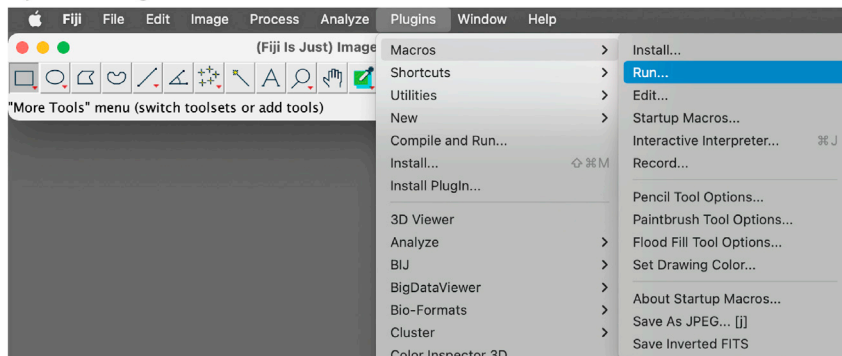
Figure 1. Home Screen View of Fiji Plugins drop-down menu
Fiji Plugins menu, with *Macros* and *Run* selected.

    a. Select *"Plugins > Macro > Run…"*.
    b. Select macro #2 in the first popup window, click *Open*.
    c. Select the desired input folder, and in the second popup window, click *Open*.
    d. Wait until the log states the conversion is complete, or the processing updates (in the bottom left corner of the Fiji window) have stopped, before moving to the next step.
    e. This will output a folder named "SplitChannel_2" into the selected input folder, which will be used as the input for CellProfiler pipeline #2.

4. In Fiji, run macro #3 (*3_Macro_pipeline3.ijm*) on image folder 3 (*3_Images_pipeline3_EdU+-FBNo*). This macro splits and pre-processes images for pipeline #3.
    a. Select *"Plugins > Macro > Run…"*.
    b. Select macro #3 in the first popup window, click *Open*.
    c. Select the desired input folder, and in the second popup window, click *Open*.
    d. Wait until the log states the conversion is complete, or the processing updates (in the bottom left corner of the Fiji window) have stopped, before moving to the next step.
    e. This will output a folder named "SplitChannel_3" into the selected input folder, which will be used as the input for CellProfiler pipeline #3.

5. In Fiji, run macro #4 (*4_Macro_pipeline4.ijm*) on image folder 4 (*4_Images_pipeline4_TUNEL*). This macro splits and pre-processes images for pipeline #4.
    a. Select *"Plugins > Macro > Run…"*.
    b. Select macro #4 in the first popup window, click *Open*.
    c. Select the desired input folder, and in the second popup window, click *Open*.
    d. Wait until the log states the conversion is complete, or the processing updates (in the bottom left corner of the Fiji window) have stopped, before moving to the next step.
    e. This will output a folder named "SplitChannel_4" into the selected input folder, which will be used as the input for CellProfiler pipeline #4.

Note: We have described Macros for splitting confocal images with 3 channels in 2 different orders (i.e., channel 1–3, representing, Green-Red-Blue or Blue-Green-Red, respectively). For confocal images with more or less than 3 channels, channel numbers and order in all Macros must be edited/updated in the Fiji macro editor to correctly split the images (by dragging the macro file into Fiji or selecting *"Plugins > Macros > Edit…"* and selecting the desired Macro in the popup window). The image-processing parameters for Macros may also need to be optimized for the user's own images, due to possible differences in image acquisition and image quality. The intention of these Macros is to achieve images similar to those seen in Figure 2, after pre-processing is complete.
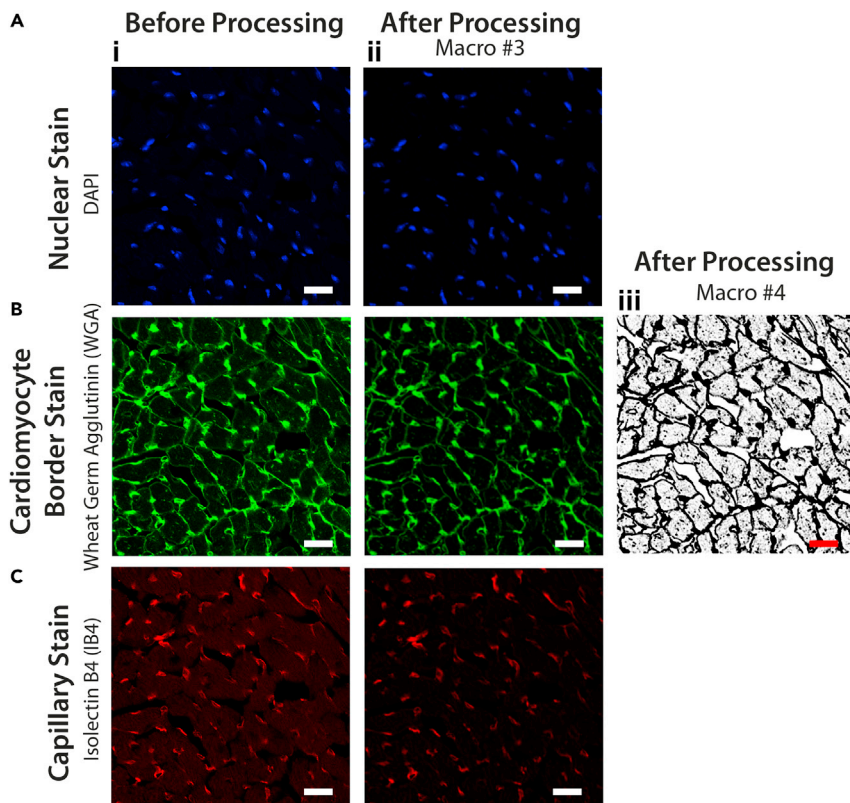
Figure 2. Representative images before and after automated processing
(A) Nuclei labeled with DAPI before (i) and after (ii) automated pre-processing steps.
(B) Cardiomyocyte boundaries labeled with Wheat Germ Agglutinin (WGA) (i), and processed in two steps (ii-iii).
(C) Capillaries labeled with Isolectin B4 (IB4) before (i) and after (ii) processing. Scale bar represents 20μm.

Analysis using CellProfiler

CellProfiler is capable of running complex image analysis pipelines on batches of several thousands of images in only a few minutes to hours. The software requires the use of analysis modules which run image processing algorithms, threshold-based segmentation, calculations and file processing. Below (Table 2), we outline the function of each module required for the segmentation of nuclei, CMs, capillaries, proliferating CFs, apoptotic cells, and immune cells in 2D confocal images from murine hearts. Table 3 can be consulted for troubleshooting problems with segmentation in CellProfiler. Pipelines optimized for three channels (Red, Green and Blue (RGB)) are listed in the key resources table.

"Settings" indicate how each module is used, while *"Functions"* outline what each module does in the context of this protocol.

Pipeline #1 – Quantify number of capillaries per cardiomyocyte
Segmentation of nuclei

⊙ Timing: 10–15 min to set optimal parameters and ~5–10 s to analyze each file with CellProfiler

Segmentation, the delineation of objects in an image, is achieved here using a number of strategies involving intensity thresholding, size thresholding, image masking and/or object relationships. The accurate segmentation of objects in tissue sections (as compared to cells in culture) is challenging

Table 2. CellProfiler modules for cardiac tissue segmentation

Module	Settings	Function
Identify Primary Objects	Global thresholding using Adaptive, Otsu approach; based on object size and intensity	Segment nuclei or whole cells (CMs and CFs)
Identify Secondary Objects	Watershed - Gradient method across the image; Adaptive, Otsu approach	Detect cell boundaries using object as seed, using primary stain (e.g., center of CM)
Identify Tertiary Objects	Subtraction	Subtract 1° object from 2° object to give 'cytosolic' area
Expand or Shrink Objects	Expand objects by 5 pixels	Expand the masked area for CM image masking
Mask Image	Select image to mask and invert the mask	Remove the background/empty space from CM images
Smooth	Smooth, keeping edges with edge intensity diff. of 0.1	Clean the immune cell images before segmentation
Overlay Objects	Select objects and image	Overlay segmented objects on image of choice
Relate Objects	Set parents (larger object) and children (smaller) - Save	Determine children (EdU$^+$ nuclei) that are segmented within parents (TdT$^+$ CFs)
Measure Colocalization	Select objects to colocalize	Determine amount of overlap between objects
Measure Image Area Occupied	Select objects to measure	Measures total area covered by cells and sum of cell perimeters
Measure Object Size Shape	Select objects to measure	Measure intensities of all cells/nuclei/cytosol
Calculate Math	Select features to define	Calculate ratio of intensity between nucleus and whole cell
Display Data on Image	Select objects and image	Number the segmented objects in the Overlaid image
Display Histogram	Select features to define	Provide histogram representation of intensity – for normal distribution
Export to Spreadsheet	Select features to export	Export all data into excel format

due to cellular complexity and structural heterogeneity present in the scar tissue. This protocol will define several analysis pipelines for the segmentation of cells in the myocardium and cardiac interstitial space, beginning with the segmentation of nuclei, labeled by DAPI.

6. Change the number of "workers" (see **Note** below) to 16 in CellProfiler to increase the parallel processing:
   a. Open CellProfiler.
   b. Select *"Menu > Preferences > Maximum Number of workers: 16"* and click *OK*.
7. Begin by loading the required CellProfiler pipeline, by either:
   a. Drag and drop the pipeline (Pipeline #1 - *1_Capillary_per_CM_segment.cpproj* in the Github repository) into the left-hand box (Figure 3A-i), below the setup tabs (Methods video S1). See **Note** below if using a later version of CellProfiler (i.e., version 4);
   b. Or, alternatively, users can build up pipelines to suit their own channel layout and experimental setup, by selecting the *"Adjust modules: +"* and selecting the modules (Figure 3A-iii), based on the logic outlined in Table 2 and the segmentation blocks in Figure 4.
8. Open pre-processed image files in CellProfiler and sort them:
   a. Select the *"Images"* tab and drag and drop image files or entire folders into CellProfiler (Figure 3A-i-ii).
   b. Select the *"NamesAndTypes"* tab (Figure 3B-i), and arrange files by selecting the rules that apply to the image names (e.g., 1. *"File > Does > Contain regular expression"* > *blue*, then 2. *"Name to assign these images"* > *DAPI*).
   c. Add another image channel by selecting *Duplicate this image* (Figure 3B-ii).
   d. Click *"Update"*, ensuring that the filenames are correctly aligned (Figure 3B-iii) (i.e., image sets are paired together).
9. Select *"View output settings"* to change the output directory to desired file location. Input setting can stay the same, as it is not being used in these pipelines.
10. Select *"Start Test Mode"* (Figure 3B-iii), this will allow the step-by-step setup of the pipeline for the user's images:
   a. Select the *"Identify Primary Objects"* module (Figure 4A-i) and ensure that *"Use advanced setting"* is checked *Yes* (Figure 4A-ii). Then identify the input image as DAPI (or another

Table 3. CellProfiler troubleshooting for segmentation

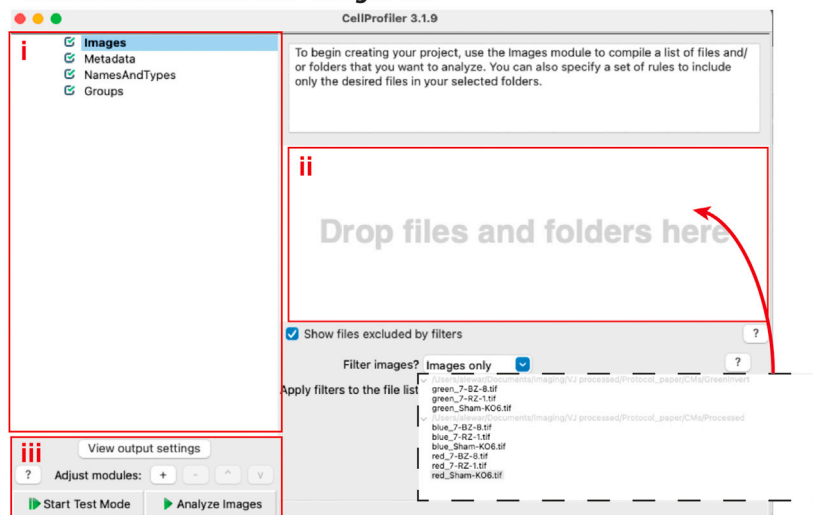| Module	Problem	Solutions
Identify Primary Objects	No objects detected	1. Adjust Min. and Max. object diameters to represent magnification (e.g., Lower for 20x images and higher for 40/63x) 2. Adjust lower threshold bound
Identify Primary Objects	Background is being segmented	Increase the lower threshold bound until only cell/tissue of interest is segmented
Identify Primary Objects	Lower intensity cells are not being segmented	1. Decrease the lower threshold bound until entire objects are detected 2. Lower Threshold correction factor
Identify Primary Objects	Many small objects are being segmented within a single cell/nucleus	Increase the Min. typical diameter of objects
Identify Primary Objects	Objects are too separated/split up (over-segmented)	1. Increase the Min. typical object diameter 2. Increase the size of smoothing filter 3. Increase local maxima distance 4. Increase the threshold correction factor 5. Change the methods to distinguish and divide clumped objects
Identify Primary Objects	Objects are merged together (under-segmented)	1. Decrease the Min. typical object diameter 2. Decrease the size of smoothing filter 3. Decrease local maxima distance 4. Decrease the threshold correction factor 5. Change the methods to distinguish and divide clumped objects
Identify Primary Objects	Objects not successfully segmented, despite changing settings above	Attempt a 3-class Otsu threshold
Identify Primary Objects	CM seed (center, within boundaries) are not detected	1. Decrease the lower threshold bound (from 0.5 to 0.2–3) 2. Change Min. and Max. object diameters 3. Potentially pre-process CMs again in Fiji to mirror representative images here

name depending on the naming convention used, select the *Question mark box* to read what each option does (Figure 4A-iii)). Nuclear segmentation should generally be performed with an *Adaptive, Otsu, Two class* threshold:

i. Set *"Object diameter in pixels"* to relatively stringent low minimum and high maximum values (e.g., Sizes = 15 and 60 pixels, respectively) (Figure 4A-iv).

ii. Set *"Lower and upper bounds on threshold"* between flexible values (e.g., 0.001–1.0), with a flexible (0.8) *"Threshold correction factor"* (Figure 4A-v).

iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0) (Figure 4A-v).

iv. Set *"Suppress Local maxima distance"* and *"Size of smoothing filter"* to lower than the minimum object size (e.g., 10 for both) (Figure 4A-vi).

v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Shape* and *Shape*, respectively (Figure 4A-vi).

b. Click *"Step"* to observe segmentation quality in the popup window with defined settings (Figure 4A-vii). If these settings are not appropriate, consult Table 2. and the CellProfiler *Question Mark box* for troubleshooting (Figure 4A-iii).

c. Once the segmentation is accurate (Figures 5A and 5B), click *"Step"* for both *"Overlay Objects"* and *"Display Data on image"*, to check segmentation and label objects for downstream analysis quality control.

d. Finally, click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.

⚠ CRITICAL: Segmentation settings should be tested on 10–20 images in the test mode prior to running finalized analysis, to ensure that they work for many images in a protocol and not just a small subset.

A **CellProfiler Home screen - Images Tab**

CellProfiler Home screen - NamesAndTypes Tab

B
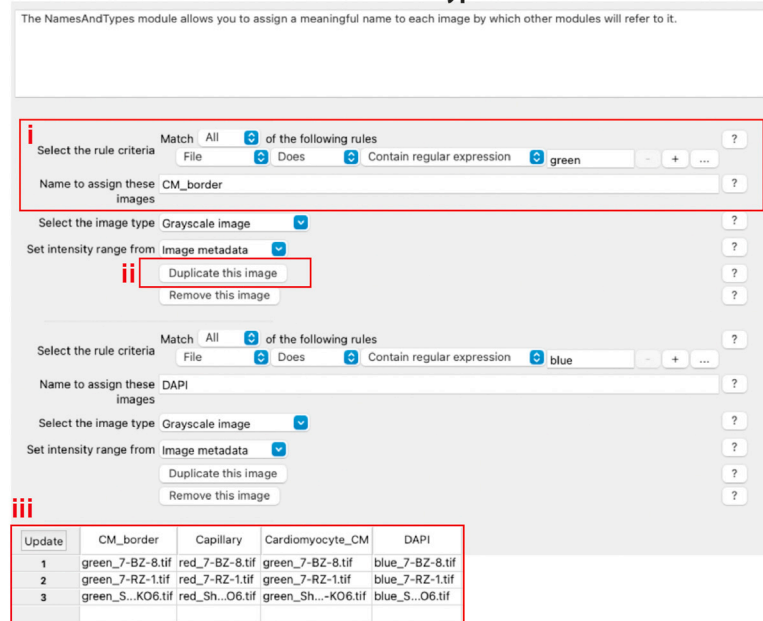
Figure 3. Home screen view of CellProfiler for setup steps
(A) The Images Tab with, (i) *Images* module selected for the importation of image files by drag and drop, and *NamesAndTypes* module for establishing rules to group images, (ii) Box showing importation of images before and after import, (iii) Box containing *View output settings* box to change output directory, Addition box for adding modules to the pipeline, *Start Test Mode* button for testing pipeline and segmentation settings, *Analyze Images* button for running final pipeline.
(B) The *NamesAndTypes* tab with, (i) rules to group image by should always use *"File > Does > Contain regular expression > Rule"*, (ii) Select *Duplicate this image* to add another channel, (iii) Update button allowing assessment of channel grouping.

Note: (1) The number of workers simply signifies the number of images that can be processed in parallel in open copies of CellProfiler. It is suggested that the numbers of workers should not exceed the number of physical cores (CPUs) in the computer being used. If using a machine with low RAM (4/8 GB), and only 8 CPUs, it may be optimal to run with only 4 or 8 workers. With the above settings, depending on the number of images to be run and the
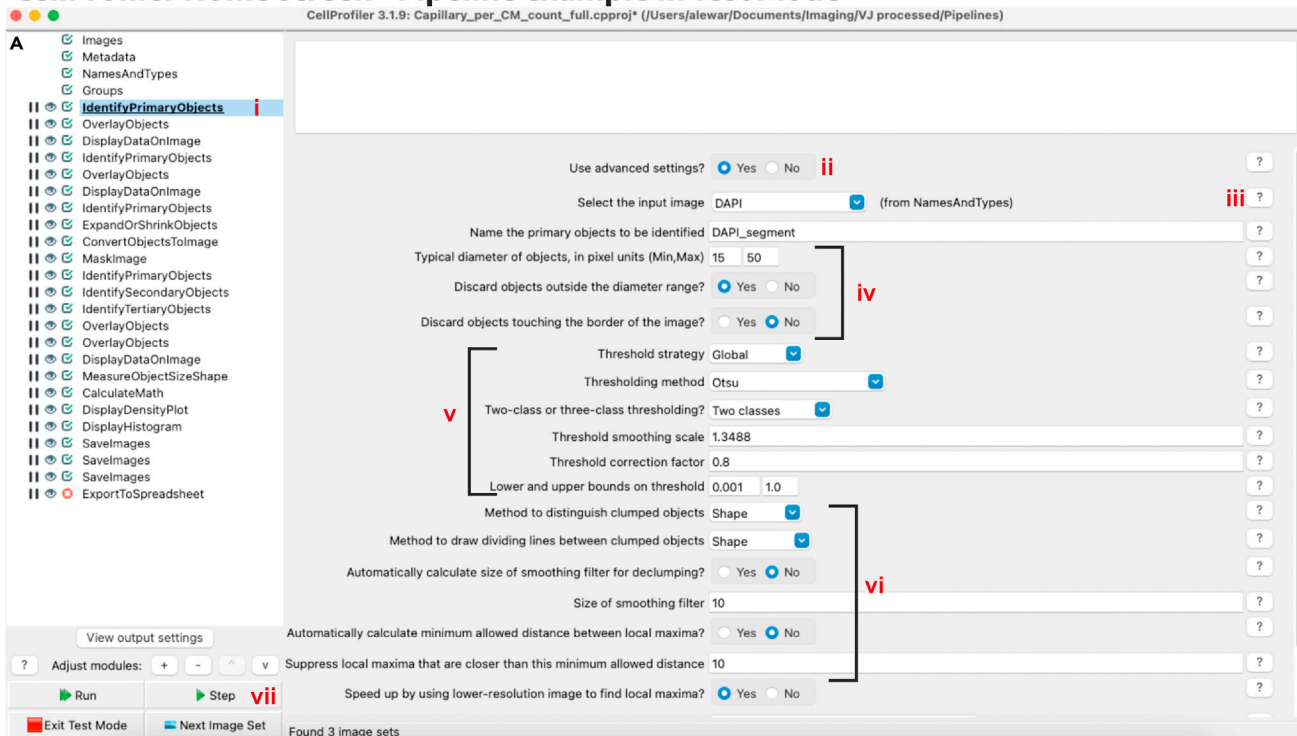
Figure 4. CellProfiler home screen view with full pipeline

(A) (i) *"IdentifyPrimaryObjects"* module for nuclei segmentation is highlighted and the following parameters correspond this module, (ii) Checkbox to use the advanced segmentation settings (check **Yes**), (iii) CellProfiler help button (*Question mark*), (iv) Size threshold parameters for the removal of objects smaller and larger than the diameter limits, (v) Intensity thresholding method and settings for segmentation, (vi) Methods for dividing segmented objects, (vii) *Step* button to progress between modules in test mode.

pipeline complexity, a lot of computer memory may be needed and up to several hours processing time (for 1000+ images). Additionally, CellProfiler generates many popup windows when performing analysis if the "eye" symbol is selected for visualization (Figure 4A-i), so it is advisable to close other programs before running segmentation tests. In the requisite pipelines, the "eye" symbols are unchecked as this helps speed up processing. (2) The CellProfiler help button (*Question Mark box* in Figure 4A-iii), gives detailed information on the function performed by each line within the module, and often the settings that can be used if objects are over-segmented (separating objects too much) or under-segmented (not separating clumped objects enough). In addition, the troubleshooting in Table 2 can be useful for the segmentation details necessary for *"Identify Primary Objects"*. (3) Each time that a module setting is changed in *"Start Test Mode"*, the module will have to be rerun.
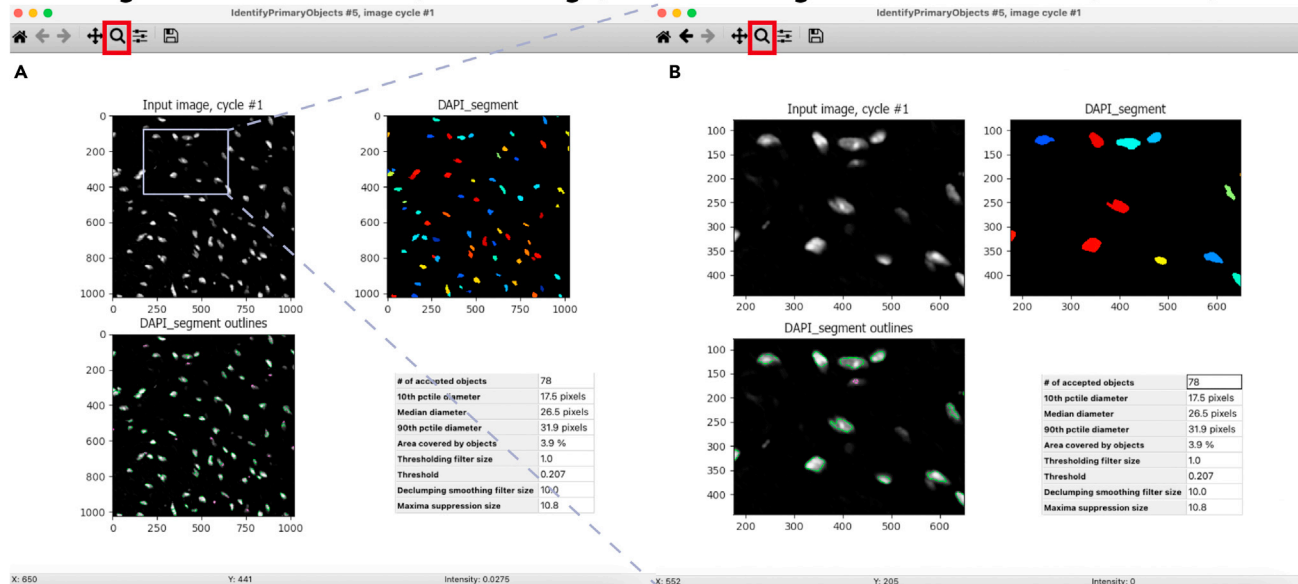
Segmentation of capillaries

⏱ Timing: 5–10 min to set optimal parameters and ~5–10 s to analyze each file with CellProfiler

11. Remaining in *"Start Test Mode"* as above:
   a. Select the next *"Identify Primary Objects"* module, with an *Adaptive, Otsu, Two class* threshold:
      i. Set *"Object diameter in pixels"* to relaxed low minimum and high maximum values (e.g., Sizes = 20 and 300 pixels, respectively).

CellProfiler "*IdentifyPrimaryObjects*" output - segmentation examples

## Segmentation of nuclei (whole image)   Segmentation of nuclei (zoomed)

Segmentation of capillaries (whole image)   Segmentation of capillaries (zoomed)
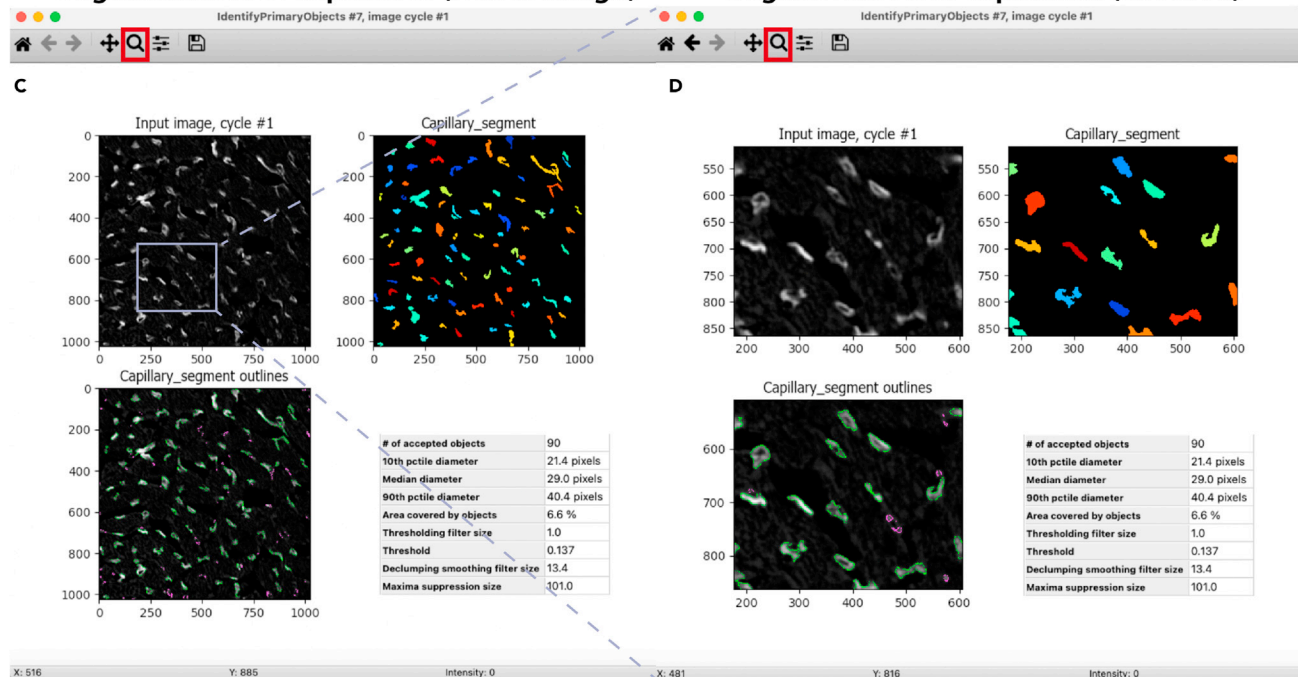
Figure 5. Representative examples of nuclei and capillary segmentation

(A and B) CellProfiler output showing representative segmentation of nuclei with box highlighting a zoomed view (B) of the same image. (C) CellProfiler output showing representative segmentation of capillaries with box highlighting a zoomed view (D) of the same image.

 ii. Set *"Lower and upper bounds on threshold"* between flexible values (e.g., 0.04–0.9), with a flexible (0.75) *"Threshold correction factor"*.

 iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).

 iv. Set *"Suppress Local maxima distance"* and *"Size of smoothing filter"* to much higher than the minimum object size (e.g., 100 for both).
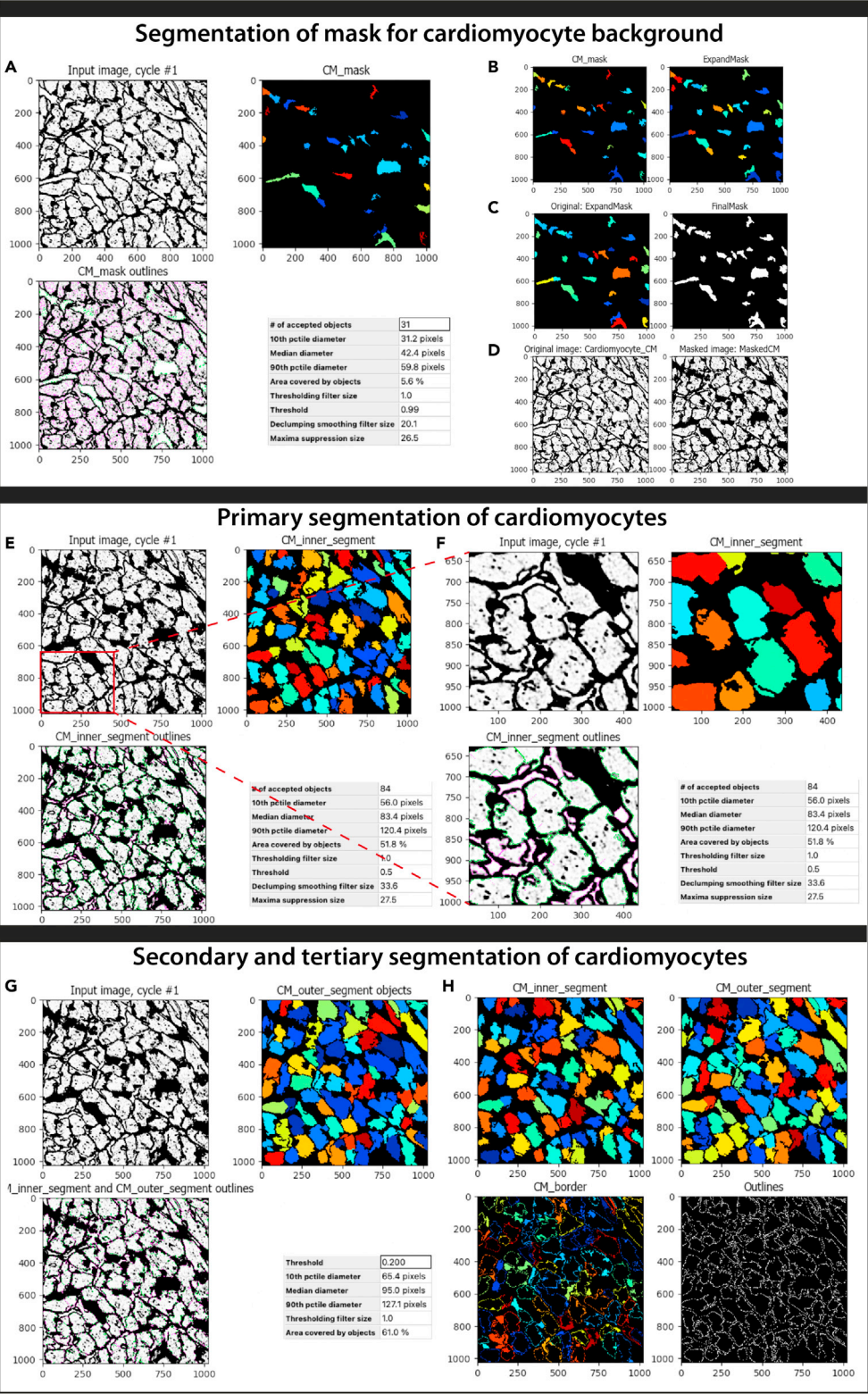
Figure 6. Representative example of CM segmentation

(A–D) Segmentation of background mask, (B) expansion of background mask, (C) generation of an image of the background mask and (D) application of the mask on the processed CM image.

Figure 6. *Continued*
(E and F) CellProfiler output showing representative segmentation of 'seed' region within CM boundaries, with box highlighting a zoomed view (F) of the same image.
(G and H) Expansion of the seed region (E) to CM boundaries and removal of the boundary region from the seed (H).
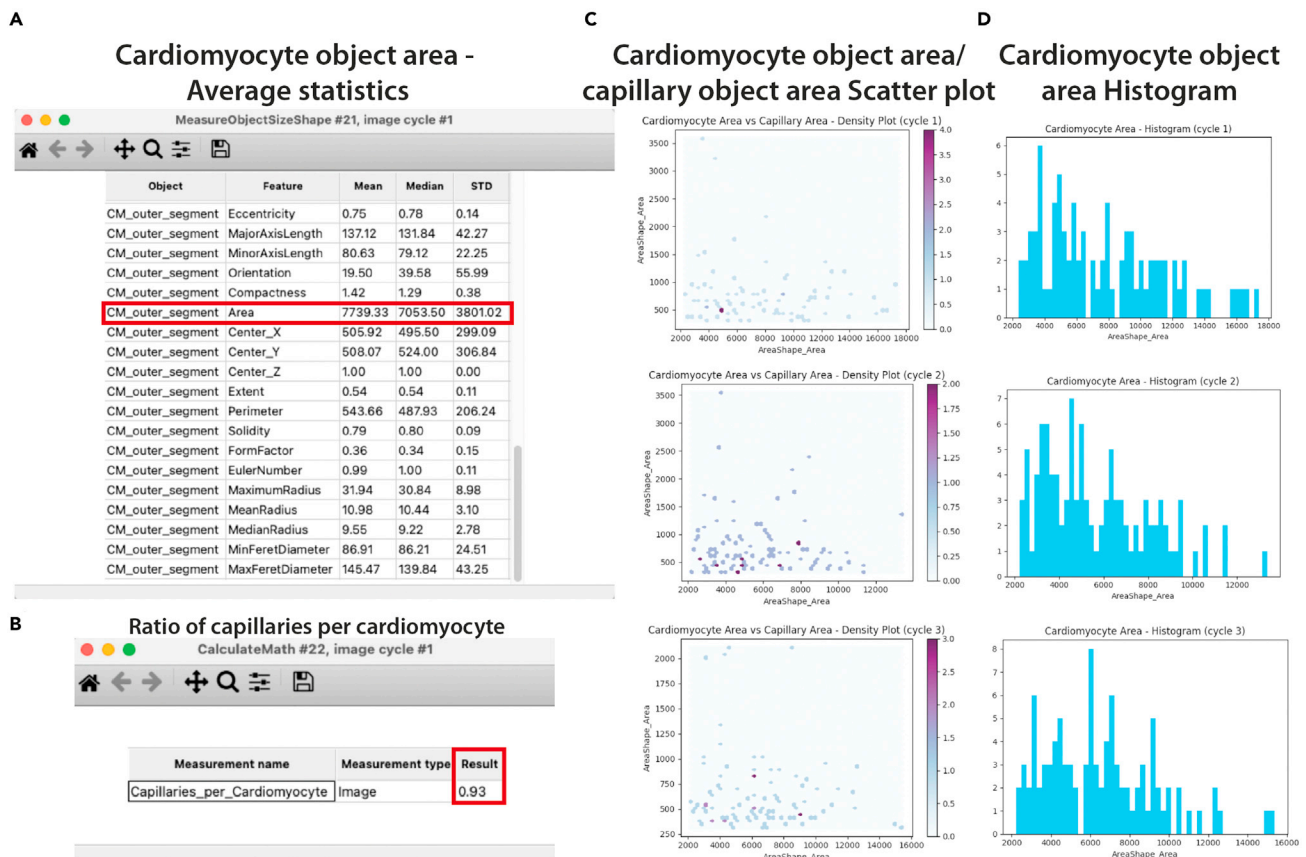
  v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Intensity* and *Shape*, respectively.
 b. Click *"Step"* to observe segmentation quality in the popup window with defined settings (Figure 4A-vii). If these settings are not appropriate, consult Table 2. and the CellProfiler *Question Mark box* for troubleshooting (Figure 4A-iii).
 c. Once the segmentation is accurate (Figures 5C and 5D), click *"Step"* for both *"Overlay Objects"* and *"Display Data on image"*, to check segmentation and label objects for downstream analysis quality control.
 d. Finally, click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.

Segmentation of CMs

⏱ Timing: ~15 min to set optimal parameters and ~20 s to analyze each file with CellProfiler

Given the larger size of CMs, nuclei of most CMs are not captured in 2D images acquired in a single plane. One common workaround to this problem is to segment CMs individually in images, rather than using segmented nuclei as a seed region and expanding to cell boundaries labeled with WGA (Figures 6A–6H).

12. Remaining in *"Start Test Mode"* as above, and using *"Step"* to sequentially test modules, first generate a mask of the blank space in the image (i.e., between myofibers).
 a. Select the next *"Identify Primary Objects"* module, with an *Adaptive, Otsu, Two class* threshold (Figure 6A):
  i. Set *"Object diameter in pixels"* to very flexible low minimum and high maximum values (e.g., Sizes = 30 and 200 pixels, respectively, to capture as much background as possible.
  ii. Set *"Lower and upper bounds on threshold"* to very stringent high values (e.g., 0.98–1.0), with a stringent (1.0) *"Threshold correction factor"* to detect solely empty background.
  iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).
  iv. *"Suppress Local maxima distance"* and *"Size of smoothing filter"* can be left to be calculated automatically.
  v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Intensity* and *Shape*, respectively.
 b. Click *"Step"* to observe segmentation quality in the popup window with defined settings.
 c. Select *"Expand or Shrink Objects"* to expand masked objects arbitrarily by a specified number of 5 pixels (Figure 6B), click *"Step"*.
 d. Select *"Convert Objects to Image"* to provide an image to mask blank space (Figure 6C), click *"Step"*.
 e. Select *"Mask Image"* to generate the blank space mask (Figure 6D), click *"Step"*.
13. Next, it is necessary to segment individual CMs, using the WGA boundaries, to perform a 3-step segmentation.
 a. First, generate a 'seed' region which is inside the WGA boundary, selecting the next *"Identify Primary Objects"* module, with an *Adaptive, Otsu, Two class* threshold (Figures 6E and 6F):
  i. Set *"Object diameter in pixels"* to relatively flexible low minimum and high maximum values (e.g., Sizes = 30–50 and 150–250 pixels, respectively.
  ii. Set *"Lower and upper bounds on threshold"* between relatively stringent values (e.g., 0.5–0.97), with a stringent (1.0) *"Threshold correction factor"*.
  iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).

Figure 7. CellProfiler graphical and statistical outputs

(A) Average statistics from CM area analysis.

(B) Quantification of numbers of capillaries per CM in representative segmented image.

(C) Scatter plot showing 3 independent examples of CM segmented areas against capillary segmented areas.

(D) Histogram showing 3 independent examples of CM segmented areas.

> iv. Set *"Suppress Local maxima distance"* and *"Size of smoothing filter"* to lower than the minimum object size (e.g., 25–30 for both).
>
> v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Intensity* and *Shape*, respectively.
>
> b. Click *"Step"* to observe segmentation quality in the popup window with defined settings.
>
> c. Next, selecting the *"Identify Secondary Objects"* module, expand the segmentation outwards from the 'seed' region, defined in 14a (Figure 6G). This will work best with an *Adaptive, Otsu, Gradient Watershed*:
>
> i. Set *"Lower and upper bounds on threshold"* between relatively flexible values (e.g., 0.2–1.0), with a stringent (1.0) *"Threshold correction factor"*
>
> ii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).
>
> d. Click *"Step"* to observe segmentation quality in the popup window with defined settings.
>
> e. Lastly, subtract the objects segmented in 14b (larger object) from 14a (smaller object), selecting the *"Identify Tertiary Objects"* module, to give the border regions (Figure 6H), click *"Step"*.
>
> f. Once the segmentation is accurate, click *"Step"* for both *"Overlay Objects"* and *"Display Data on image"*, to check segmentation and label objects for downstream analysis quality control.

14. To quantify the area and sizes of CMs (for hypertrophy studies), or determine the numbers of capillaries present per CM (for rarefaction studies), the following quantification modules can be used (Figures 7A–7D):

  a. Select the *"Measure Object Size Shape"* module, to quantify geometric parameters of segmented capillaries (from 12b) and cardiomyocytes (from 14b) (Figure 7A), click *"Step"*.

  b. Select the *"Measure Image Area Occupied"* module, to quantify geometric parameters of segmented capillaries (from 12b) and cardiomyocytes (from 14b), click *"Step"*.

  c. Select the *"Calculate Math"* module, to quantify the ratio of capillaries to cardiomyocytes (Figure 7B) (outputs to "Image" .csv file), click *"Step"*.

  d. Use the *"Display Density Plot"* module, to show the shared distribution of capillary and cardiomyocyte areas (Figure 7C), click *"Step"*.

  e. Select the *"Display Histogram"* module, to show the distribution of cardiomyocyte areas (Figure 7D), click *"Step"*.

15. Finally, click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.

16. Once all segmentation steps are optimized, the pipeline is ready to be run, select *"Analyse Images"*.

17. Once the run is complete, data, in .csv file format, and segmentation images, in .tiff file format, will be available in the output directory for downstream processing.

Note: (1) Once *"Analyze Images"* is clicked to run the pipeline, there is often a delay before the analysis starts. Depending on the specification of the computer used and the number of images analyzed, this may vary from 1–10 min. At this point, it is advisable to be patient and wait for analysis to begin. If 10 minutes has passed, there may be an error and CellProfiler may need to be restarted. (2) Following the analysis of the users' own images, it is advisable to cross check all of the segmentation example .tiff files in the output directory for accurate segmentation and quantification. Due to the inherent variability in the acquisition of microscopy images, the settings may not be applicable for every image in the image set, and as such, it may be necessary to run separate analyses in CellProfiler, with changed settings, on those images not properly segmented. (3) There are additional quantification modules that may be useful for determining features of interest, such as the proximity of CMs to one another (*"Measure Object Neighbors"*) or internal features of myocytes (*"Measure Texture"* or *"Measure Granularity"*). However, these may only be useful for a specific purpose. For the more standardized cardiac phenotype indexing, CM hypertrophy and capillary rarefaction should be sufficient. (4) Depending on the number of images to be run and the pipeline complexity, this may require a lot of computer memory, and up to several hours (for 1000+ images).

Pipeline #2 – Quantify number of immune cells per cardiomyocyte
Segmentation of immune cells

⏱ Timing: 5–10 min to set optimal parameters and ∼5–10 s to analyze each file with CellProfiler

The irregular shape and projections of invading immune cells (CD45^{+}) in the cardiac interstitium make them inherently challenging to segment. Here, we describe an additional CellProfiler pre-processing step to clean the edges and boundaries of the CD45^{+} cells analyzed.

18. Begin by loading the required CellProfiler pipeline, by either:

  a. Drag and drop the pipeline (Pipeline #2 - *2_CD45_per_CM_segment.cpproj* in the Github repository) into the left-hand box (Figure 3A-i), below the setup tabs.

  b. Or, alternatively, users can build up pipelines to suit their own channel layout and experimental setup, by selecting the *"Adjust modules: +"* and selecting the modules (Figure 3A-iii), based on the logic outlined in Table 2 and the segmentation blocks in Figure 4.

19. Open pre-processed image files in CellProfiler and sort them:

  a. Select the *"Images"* tab and drag and drop image files or entire folders into CellProfiler (Figure 3A-i-ii).
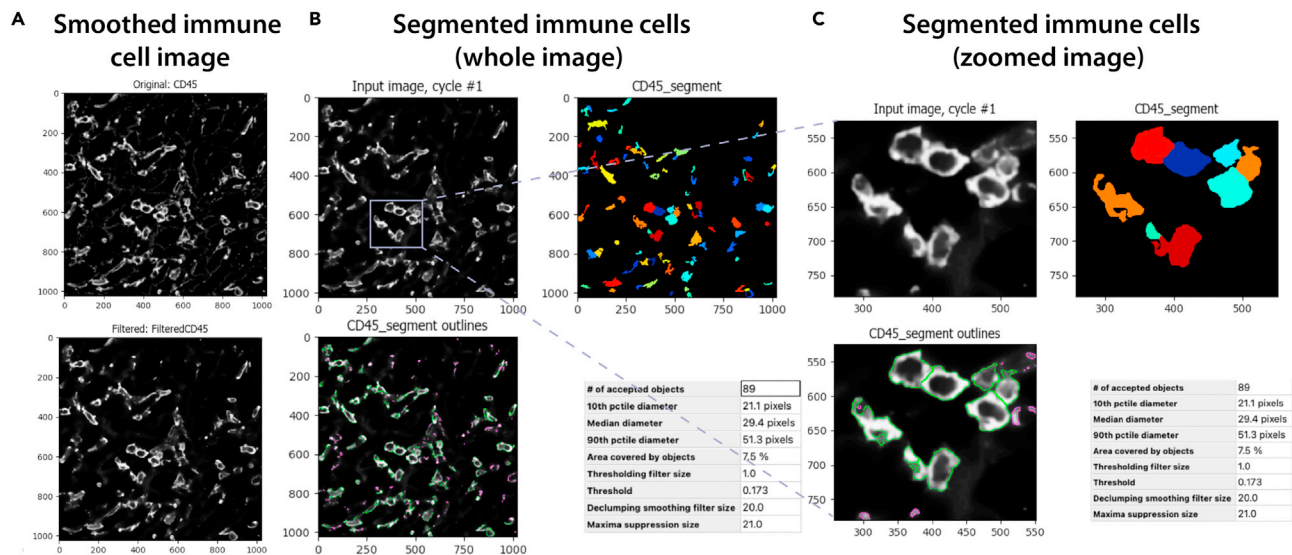
Figure 8. Representative examples of immune cell processing and segmentation

(A) CellProfiler output showing immune cell image before and after smoothing.

(B and C) CellProfiler output showing representative segmentation of smoothed immune cells with box highlighting a zoomed view (C) of the same image.

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  b. Select the *"NamesAndTypes"* tab (Figure 3B-i), and arrange files by selecting the rules that apply to the image names (e.g., 1. *"File > Does > Contain regular expression" > blue*, then 2. *"Name to assign these images" > DAPI*).

  c. Add another image channel by selecting *Duplicate this image* (Figure 3B-ii).

  d. Click *"Update"*, ensuring that the file names are correctly aligned (Figure 3B-iii) (i.e., channels from each image set are paired up).

20. Select *"View output settings"* to change the output directory to desired file location. Input setting can stay the same, as it is not being used in these pipelines.

21. Enter *"Start Test Mode"* as above.

22. Stepwise, progress through the nuclei segmentation steps, from the first *"Identify Primary Objects"* module, as above (Step 10).

23. After running the first *"Save Images"* module, proceed forward with CD45^{+} cell segmentation below:

  a. Select *"Smooth"* module to homogenize the appearance of CD45^{+} cells, using the function *Smooth keeping edges*, and automatically calculating the *Artifact diameter*. Keep the *Edge Intensity difference* at 0.1 (Figure 8A), click *"Step"*.

  b. Segmentation of CD45^{+} cells is then performed by selecting the *"Identify Primary Objects"* module, using an *Adaptive, Otsu, Two class* threshold:

   i. Set *"Object diameter in pixels"* to relatively stringent low minimum and high maximum values (e.g., Sizes = 20 and 100 pixels, respectively.

   ii. Set *"Lower and upper bounds on threshold"* between flexible values (e.g., 0.005–1.0), with a flexible (0.75) *"Threshold correction factor"*.

   iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).

   iv. Set *"Suppress Local maxima distance"* and *"Size of smoothing filter"* to lower than the minimum object size (e.g., 20 for both).

   v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Shape* and *Shape*, respectively.

  c. Click *"Step"* to observe segmentation quality in the popup window with defined settings.

  d. Once the segmentation is accurate (Figures 8B and 8C), click step for both *"Overlay Objects"* and *"Display Data on image"* modules, to check segmentation and label objects for
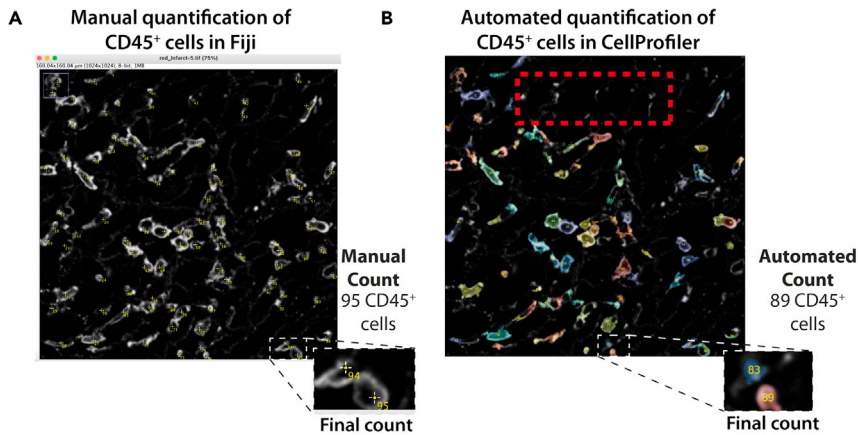
Figure 9. Comparison of manual and automated segmentation methods for immune cell quantification
(A and B) Manual quantification of immune cells using Fiji compared with the automated quantification (B) by CellProfiler. The red box identifies a region of the image, where objects were not segmented due to them being below the intensity threshold. This highlights the necessity for correct intensity thresholding across many images.

downstream analysis quality control, such as with a comparison against manual CD45$^+$ cell quantification with Fiji (Figure 9).

24. Finally, click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.
25. Stepwise, progress through the CM segmentation steps, as above (steps 13–16).
26. Once all segmentation steps are optimized, the pipeline is ready to be run, select *"Analyse Images"*.
27. Once the run is complete, data, in .csv file format, and segmentation images, in .tiff file format, will be available in the output directory for downstream processing.

Pipeline #3 – Quantify number of proliferating fibroblasts
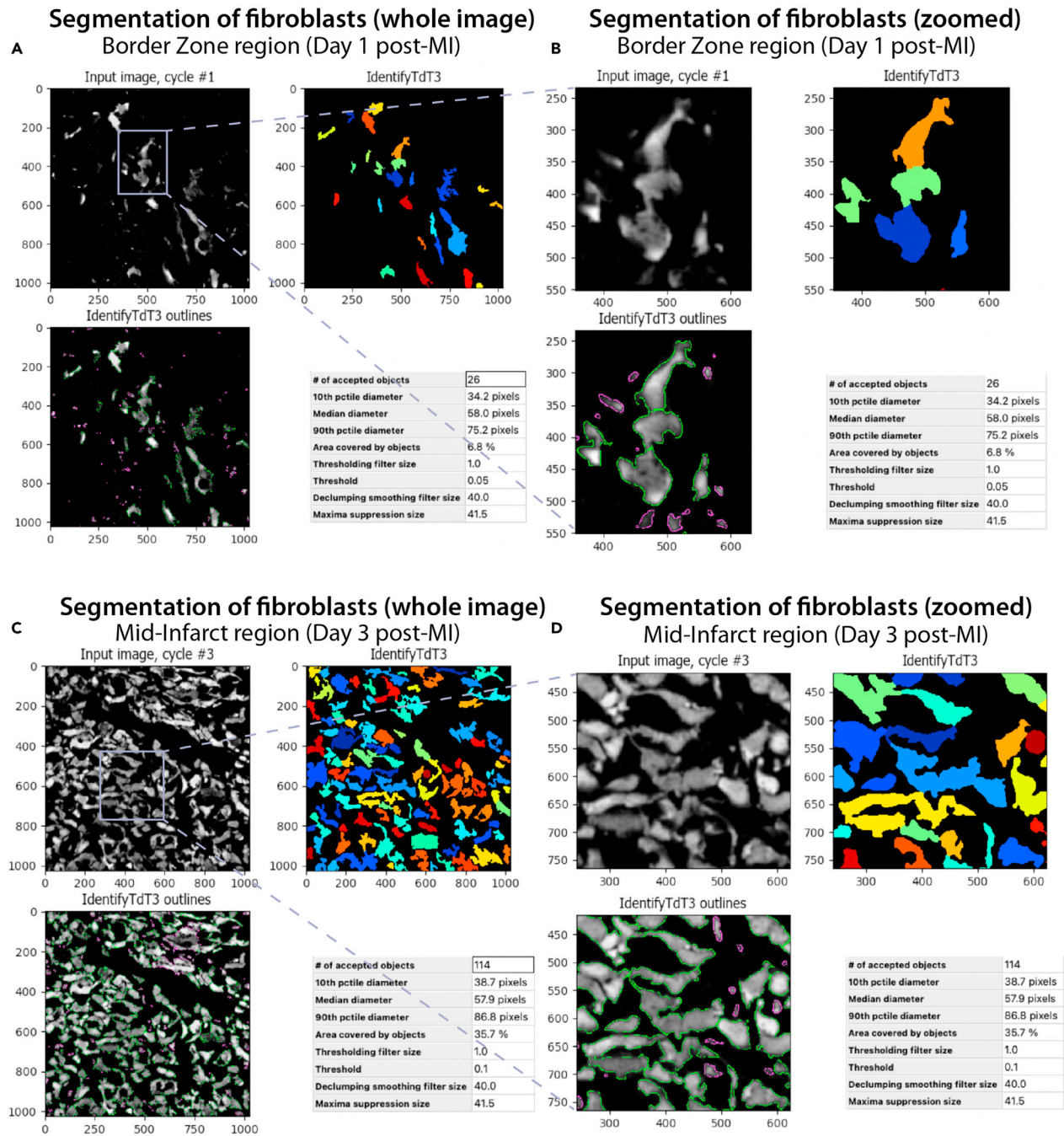*Segmentation and quantitation of proliferating CFs*

⊙ Timing: 5–10 min to set optimal parameters and ∼5–10 s to analyze each file with CellProfiler

28. Begin by loading the required CellProfiler pipeline, by either:
    a. Drag and drop the pipeline (Pipeline #3 - *3_EdU_FB_segment.cpproj* in the Github repository) into the left-hand box (Figure 3A-i), below the setup tabs.
    b. Or, alternatively, users can build up pipelines to suit their own channel layout and experimental setup, by selecting the *"Adjust modules: +"* and selecting the modules (Figure 3A-iii), based on the logic outlined in Table 2 and the segmentation blocks in Figure 4.
29. Open pre-processed image files in CellProfiler and sort them:
    a. Select the *"Images"* tab and drag and drop image files or entire folders into CellProfiler (Figure 3A-i-ii).
    b. Select the *"NamesAndTypes"* tab (Figure 3B-i), and arrange files by selecting the rules that apply to the image names (e.g., 1. *"File > Does > Contain regular expression"* > *blue*, then 2. *"Name to assign these images"* > *DAPI*).
    c. Add another image channel by selecting *Duplicate this image* (Figure 3B-ii).
    d. Click *"Update"*, ensuring that the filenames are correctly aligned (Figure 3B-iii) (i.e., image sets are paired together).
30. Select *"View output settings"* to change the output directory to desired file location. Input setting can stay the same, as it is not being used in these pipelines.
31. Enter *"Start Test Mode"* as above.

32. Stepwise, progress through the nuclei segmentation steps, from the first "*Identify Primary Objects*" module, as above (Step 10).

33. After running the first "*Save Images*" module, proceed forward with CF TdT$^+$ cell segmentation below:

    a. Select "*Identify Primary Objects*" module, segment with an *Adaptive, Otsu, Three class* threshold:

       i. Set "*Object diameter in pixels*" to relatively stringent low minimum and high maximum values (e.g., Sizes = 30 and 120 pixels, respectively).

       ii. Set "*Lower and upper bounds on threshold*" between flexible values (e.g., 0.05–1.0), with a flexible (0.75) "*Threshold correction factor*".

       iii. Set "*Threshold smoothing scale*" to 1.3488 (Sigma of 1.0).

       iv. "Assign pixels in the middle intensity class to the: Background".

       v. Set "*Suppress Local maxima distance*" and "*Size of smoothing filter*" to higher than the minimum object size (e.g., 40 for both).

       vi. Finally, set "*Method to distinguish clumped objects*" and "*Method to draw dividing lines between clumped objects*" to *Intensity* and *Shape*, respectively.

    b. Click "*Step*" to observe segmentation quality in the popup window with defined settings.

    c. Once the segmentation is accurate (Figures 10A–10D), click "*Step*" for both "*Overlay Objects*" and "*Display Data on image*" modules, to check segmentation and label objects for downstream analysis quality control.

34. Segmenting EdU$^+$ nuclei requires the same process as Step 10a-c (Segmentation of nuclei):

    a. Select the "*Identify Primary Objects*" module and segment with an *Adaptive, Otsu, Two class* threshold:

       i. Set "*Object diameter in pixels*" to relatively stringent low minimum and high maximum values (e.g., Sizes = 15 and 60–80 pixels, respectively).

       ii. Set "*Lower and upper bounds on threshold*" between flexible values (e.g., 0.001–1.0), with a flexible (0.8) "*Threshold correction factor*".

       iii. Set "*Threshold smoothing scale*" to 1.3488 (Sigma of 1.0).

       iv. Set "*Suppress Local maxima distance*" and "*Size of smoothing filter*" to lower than the minimum object size (e.g., 10 for both).

       v. Finally, set "*Method to distinguish clumped objects*" and "*Method to draw dividing lines between clumped objects*" to *Shape* and *Shape*, respectively.

    b. Click "*Step*" to observe segmentation quality in the popup window with our defined settings.

    c. Once the segmentation is accurate, click "*Step*" and perform both "*Overlay Objects*" and "*Display Data on image*", to quality check segmentation and label objects for downstream analysis quality control.

35. To first quantitate the numbers of proliferating CFs, select the "*Measure colocalization*" module and select EdU and CF images for comparison, and compare with the threshold set at 10% of the maximum intensity across the entire image. Check *Yes* for "*Run all metrics*" option.

36. Additionally, it is necessary to quantify overlapping segmented CFs with segmented EdU$^+$ nuclei. To do this, the segmented objects must be related to one another (i.e., EdU$^+$ nuclei that lie within segmented CF areas) and are then re-counted (Figures 11A and 11B):

    a. Firstly, select the "*Relate Objects*" module, with segmented CFs as the "*Parent objects*" and segmented EdU$^+$ nuclei as the "*Child objects*", click "*Step*".

    b. Save the children with parents as a new object set (check *Yes*).

    c. Select "*Convert objects to image*" to save the related EdU$^+$ CFs (double positive) objects as a new *Colour* image, click "*Step*".

    d. Once the segmentation is accurate, click "*Step*" for both "*Overlay Objects*" and "*Display Data on image*", to check segmentation and label objects for downstream analysis quality control.

37. Lastly, quantify parameters relevant to amounts of double positivity and CF sizes.

    a. Select the "*Measure Object Size Shape*" module, to quantify geometric parameters of segmented CFs, click "*Step*".

Segmentation of fibroblasts (whole image)
Border Zone region (Day 1 post-MI)

Segmentation of fibroblasts (zoomed)
Border Zone region (Day 1 post-MI)

Segmentation of fibroblasts (whole image)
Mid-Infarct region (Day 3 post-MI)

Segmentation of fibroblasts (zoomed)
### Mid-Infarct region (Day 3 post-MI)

Figure 10. Representative examples of CF segmentation at different stages after Myocardial Infarction

(A and B) CellProfiler output showing representative segmentation of CFs from the border zone 1-day post-MI with box highlighting a zoomed view (B) of the same image.

(C and D) CellProfiler output showing representative segmentation of CFs from the mid-infarct region 3-days post-MI with box highlighting a zoomed view (D) of the same image.

b. Select the "*Measure Image Area Occupied*" module, to quantify geometric parameters of segmented CFs, click "*Step*".

c. Select the "*Calculate Math*" module, to quantify the percentage of proliferating CFs (outputs to "Image" .csv file), click "*Step*".

A

B
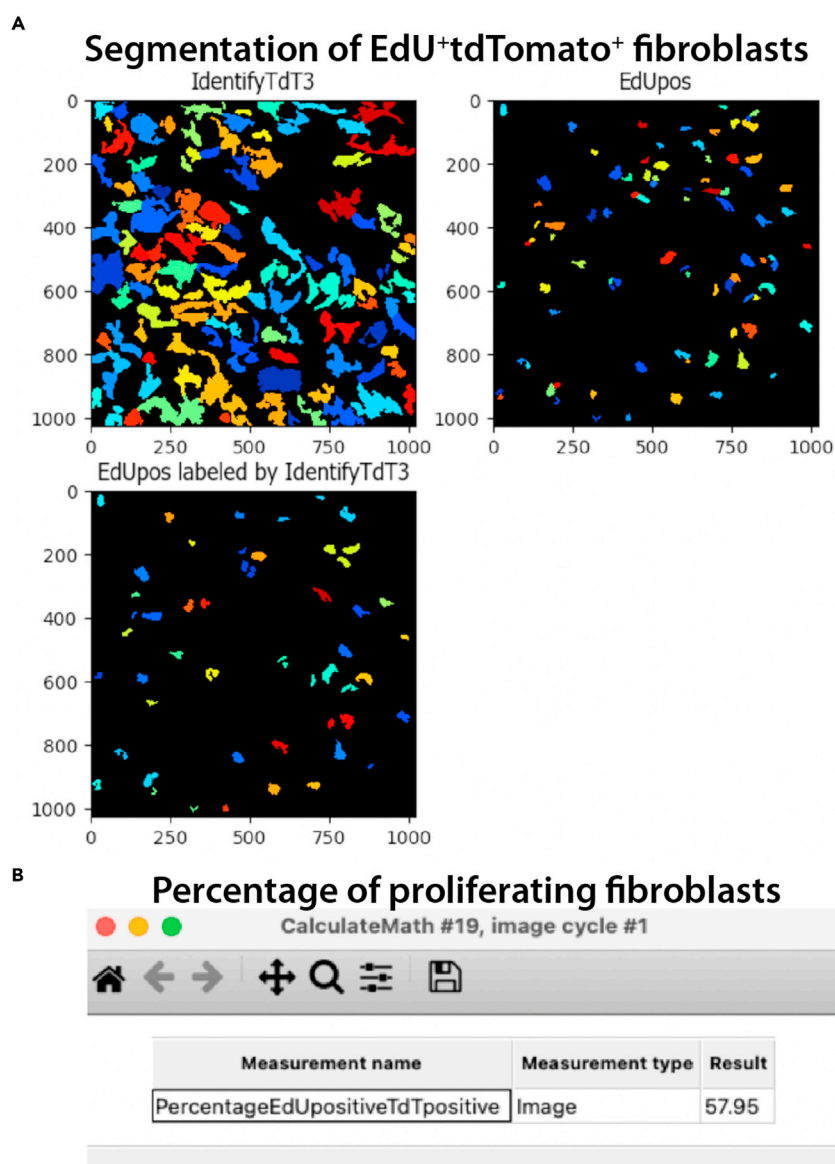
Figure 11. Example relating segmented proliferating nuclei with segmented CFs
(A and B) CellProfiler example of the related objects process and the quantification (B) showing the percentage of active proliferating CFs of the total segmented CFs.

38. Finally, click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.
39. Once all segmentation steps are optimized, the pipeline is ready to be run, select *"Analyse Images"*.
40. Once the run is complete, data, in .csv file format, and segmentation images, in .tiff file format, will be available in the output directory for downstream processing.

Note: The quantification of proliferating CFs was performed here; however, it is conceivable also to quantify total proliferation or other cell-type proliferation by modifying the modules within the pipeline as necessary.

Pipeline #4 – Quantify number of apoptotic cells
Segmentation of apoptotic nuclei

> ⏱ Timing: 5–10 min to set optimal parameters and ~5–10 s to analyze each file with CellProfiler

TUNEL staining is used to detect DNA breaks formed during the final phase of apoptosis, when DNA fragmentation takes place.

41. Enter *"Start Test Mode"* as above.
42. Stepwise, progress through the nuclei segmentation steps, from the first *"Identify Primary Objects"* module, as above (Step 10).
43. After running the first *"Save Images"* module, proceed forward with TUNEL⁺ cell segmentation below.
44. Segmenting TUNEL⁺ nuclei require the same process as Step 10a-c (Segmentation of nuclei):
    a. Select the *"Identify Primary Objects"* module. Nuclear segmentation should generally be performed with an *Adaptive, Otsu, Two class* threshold:
        i. Set *"Object diameter in pixels"* to relatively stringent low minimum and high maximum values (e.g., Sizes = 15 and 60–80 pixels, respectively).
        ii. Set *"Lower and upper bounds on threshold"* between flexible values (e.g., 0.001–1.0), with a flexible (0.8) *"Threshold correction factor"*.
        iii. Set *"Threshold smoothing scale"* to 1.3488 (Sigma of 1.0).
        iv. Set *"Suppress Local maxima distance"* and *"Size of smoothing filter"* to lower than the minimum object size (e.g., 10 for both).
        v. Finally, set *"Method to distinguish clumped objects"* and *"Method to draw dividing lines between clumped objects"* to *Shape* and *Shape*, respectively.
    b. Click *"Step"* to observe segmentation quality in the popup window with our defined settings. If these settings are not appropriate, consult Table 2. and the CellProfiler *Question Mark box* for troubleshooting.
    c. Once the segmentation is accurate, click *"Step"* for both *"Overlay Objects"* and *"Display Data on image"*, to check segmentation and label objects for downstream analysis quality control.
45. Click *"Step"* on the *"Save Images"* module to save segmented images as numbered overlays.
46. Additionally, it is necessary to quantify overlapping segmented nuclei with segmented TUNEL⁺ nuclei. To do this, the segmented objects must be related to one another (i.e., TUNEL⁺ nuclei that lie within segmented nuclear areas) and are then re-counted:
    a. Firstly, select the *"Relate Objects"* module, with segmented nuclei as the *"Parent objects"* and segmented TUNEL⁺ nuclei as the *"Child objects"*, click *"Step"*.
    b. Save the children with parents as a new object set (check *Yes*).
    c. Select *"Convert objects to image"* to save the related TUNEL⁺ DAPI⁺ (double positive) objects as a new *Colour* image, click *"Step"*.
    d. Once the segmentation is accurate, click *"Step"* for both *"Overlay Objects"* and *"Display Data on image"*, to check segmentation and label objects for downstream analysis quality control.
47. Once all segmentation steps are optimized, the pipeline is ready to be run, select *"Analyse Images"*.
48. Once the run is complete, data, in .csv file format, and segmentation images, in .tiff file format, will be available in the output directory for downstream processing.

> *Note:* Segmentation of apoptotic nuclei can be performed for any cell-type specific segmented objects (e.g., CFs, CMs, IB4⁺ capillaries or CD45⁺ cells), as performed in Step 46, via the *"Relate Objects"* module.

EXPECTED OUTCOMES

Studying cardiac parameters in infarcted mouse hearts is technically challenging owing to the cellular complexity and structural heterogeneity present in the scar tissue. Our protocol overcomes the challenges associated with manual handling and user-based decisions, and therefore improves the reliability of morphological comparisons. Thus, this semi-automated computational approach improves objectivity by minimizing bias associated with manual image processing and quantification. With this protocol, cardiac parameters can be reliably quantified in infarcted mouse hearts and in different anatomical locations, considerably faster than manual assessment. Additionally, immune cells can be differentially labeled to discern monocyte and macrophage sub-populations (e.g., M1 and M2), and quantified using this pipeline. Data generated using this protocol has recently been published (Janbandhu et al., 2021). While we have used this protocol for studies mainly in infarcted mouse hearts, this pipeline can easily be applied to the other tissue types.

QUANTIFICATION AND STATISTICAL ANALYSIS

The output file (.csv) can be imported into most of the commonly used data analysis software such as Excel and GraphPad Prism. Output files contain many redundant columns, unnecessary for analysis. Generally, the columns relating to cell counts, shape and size, and other morphological parameters are the only necessary columns (see the annotated .xlsx file to determine which columns are of use for analysis). Statistical analysis can be performed on the absolute or relative measurements as required. Objects not segmented properly can be determined from the overlay output images, these should subsequently be excluded from the analysis.

LIMITATIONS

This protocol is optimized for quantifying parameters essential for the functioning of the cardiac interstitium in healthy and infarcted murine hearts. This represents an automated pipeline to quickly and quantitatively measure the cell-type, size and distribution in the murine myocardium and interstitial regions from 2D fluorescent images. As with many image analysis processes, automated segmentation of tissue requires a balance between over- and under-segmentation. Due to atypical cell morphologies *in vivo*, it may not be possible to capture every single cell in an image field of view with hard size and intensity thresholding methods, as used here. Inherently, when segmenting immune cells and fibroblasts in tissues, which are typically amorphous cell-types crowded into the interstitial space, there will be cases of imperfections in segmentation (cells split or clumped when they should not be) which are unavoidable. Given the nature of automation, however, errors will be identical between images; as opposed to those from manual segmentation methods, which are open to investigator-bias. This pipeline aims to reduce segmentation and quantification errors to within 5% of those seen with a manual segmentation method.

TROUBLESHOOTING

This table outlines some potential solutions for segmentation problems that can arise in CellProfiler. Solutions are sometimes numbered in order of priority, so that users can trial each adjustment in test mode. Additionally, the question mark box next to the parameter has useful tips for segmentation.

Problem 1

Channels are split incorrectly by Fiji Macros (steps 2–5)

Potential solution

Input the raw image into Fiji and run the *"Image > Color > Split Channels"* function. Check the channel number given to each image name in Fiji (e.g., C1 = Green Channel) (See Figure 1). If this does not match up with the name given to the channel in Macros, alter the color to match the number. It may be useful to compare Macros #1 or 2, to determine how the macro layout relates to channel order.

Problem 2

Poor segmentation of cells from injured zones of the heart (steps 12–17 and 33–38)

Potential solution

Injured regions show drastically different cell morphology and distribution, due to scarring, as such, these regions may not always segment well. Settings may need to be adjusted to segment images from within the infarcted zone, and these images run separately from non-injured myocardium.

Problem 3

Segmentation of cardiomyocytes is incomplete (steps 12–17)

Potential solution

This may occur if cardiomyocyte images have been 'over-enhanced' in the pre-processing steps and is easily corrected by modifying the pixel saturation value in Macro #1,2 and 4. If cardiomyocyte boundaries are too dark, lower this value. If they are not dark enough, increase this value.

Problem 4

Segmentation of individual objects is still unsatisfactory (under- or over-segmented) due to poor input image quality (relevant to each pipeline - steps 6–50)

Potential solution

It is possible that, if the initial image quality is sub-optimal, CellProfiler will be unable to segment single objects properly. For instance, discrimination between a group of clumped objects may be impossible to resolve due to high background or blur in the image (from widefield fluorescence images). This may be unresolvable for segmentation of individual objects; however, data may be presented as a total area occupied by objects, thus overcoming the issue of object segmentation as the image segmentation should still be able to discern gross background from foreground based on intensity values.

Problem 5

Very slow processing of images or pipeline does not seem to be progressing (steps 16, 26, 39 and 50)

Potential solution

Occasionally, after *"Analyze Images"* is clicked, CellProfiler does not respond. This may be due to the visualization being left on ("eye" symbol checked), too many images being processed and/or another fault. In this case, there is no benefit to waiting for the pipeline to respond, so, providing the pipeline is saved, the user should just close CellProfiler and reopen it.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard P. Harvey (r.harvey@victorchang.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Janbandhu et al. (2021) contains representative datasets as would typically be obtained using this protocol. In addition, images, Macros and analysis pipelines will be available in the Victor Chang Cardiac Research Institute Github repository: https://github.com/VCCRI/Cardiac-Image-Analysis. git.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101097.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology and Investigation, A.O.W, G.C. and V.J.; Writing – Original Draft, A.O.W and V.J.; Writing – Review & Editing, All Authors; Funding Acquisition and Supervision, V.J., S.L.D. and R.P.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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