

Read-out/Read-in 1.0 User Guide

Step-by-Step Instructions on Using Matlab Script readoutreadin.m

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Multiplexed in situ hybridization chain reaction (HCR) enables accurate and precise relative quantitation of mRNA expression with subcellular resolution within whole-mount vertebrate embryos (Trivedi *et al.*, 2018). Gene expression can be queried in two directions: read-out from anatomical space to expression space reveals co-expression relationships in selected regions of the specimen; conversely, read-in from multidimensional expression space to anatomical space reveals those anatomical locations in which selected gene co-expression relationships occur. This User Guide provides step-by-step instructions on using the Matlab script `readoutreadin.m` to perform read-out/read-in analyses and associated subcircuit analyses.

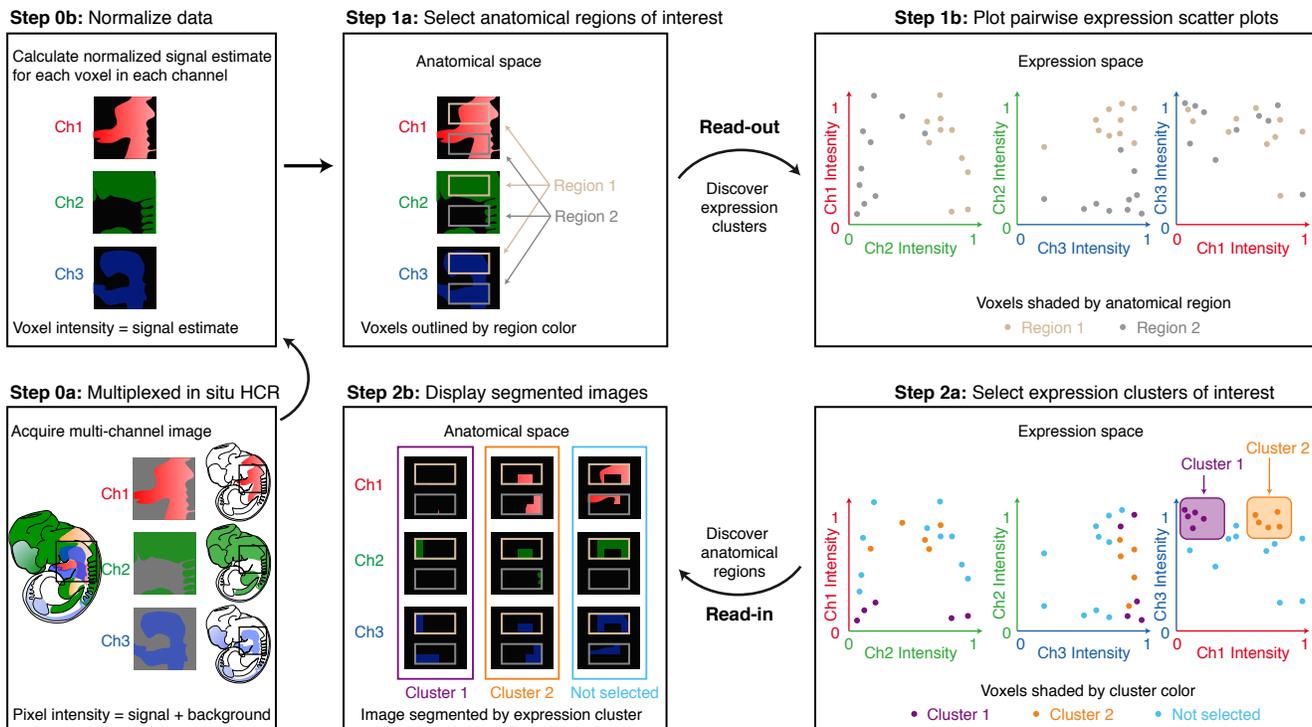


Figure 1. Work flow for quantitative read-out and read-in analyses using in situ HCR (Trivedi *et al.*, 2018). Step 0: Acquire and normalize data. Step 1: Read-out from anatomical space to expression space. Step 2: Read-in from expression space to anatomical space.

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Overview of Read-out/Read-in Workflow

See Figure 1 for an illustration of the read-out/read-in workflow:

Step 0: Acquire and normalize data

- a) **Perform a multiplexed in situ HCR experiment.** Image the expression patterns for N target mRNAs using an N -channel experiment in a single specimen (Choi *et al.*, 2016) (3 channels depicted in Figure 1). HCR probe sets, amplifiers, and buffers can be obtained from Molecular Instruments (molecularinstruments.org), a non-profit academic resource within the Beckman Institute at Caltech.
- b) **Normalize data.** Average pixel intensities to create raw voxel intensities for each channel (Section S1.4.1 of Trivedi *et al.* (2018)) and then calculate the normalized signal estimate for each voxel in each channel (Section S1.4.3 of Trivedi *et al.* (2018)). The same normalization should be applied to all images to enable quantitative comparison between embryos.

Step 1: Read-out from anatomical space to expression space

- a) **Select anatomical regions of interest.** 2 regions of interest depicted in Figure 1 (tan and gray rectangles).
- b) **Plot pairwise expression scatter plots.** For each pair of channels, plot an expression scatter plot displaying the normalized signal intensity for each voxel in the regions of interest. Dots are shaded by region color (tan or gray dots in Figure 1; see also examples in Figure 4C and 5B of Trivedi *et al.* (2018)).

Step 2: Read-in from expression space to anatomical space

- a) **Select expression clusters of interest.** Dots are shaded by expression cluster (magenta and orange clusters selected in Ch3 vs Ch1 scatter plot, dots not selected shaded blue).
- b) **Display segmented image.** Redisplay the multichannel image showing only the voxels in one expression cluster at a time (images bounded by magenta, orange, or blue rectangles; see also example of Figure 4D of Trivedi *et al.* (2018)).

Step-by-Step Instructions for Read-out/Read-in Analyses and Associated Subcircuit Analyses

Here we provide step-by-step instructions on using the Matlab script `readoutreadin.m` to perform read-out/read-in analyses and associated subcircuit analyses. We provide a sample image for testing the script (`zfish.tif`: the 4-channel image that was used to prepare Figures 4 and 5 of Trivedi *et al.* (2018)) and a set of sample output files in the directory `sample-output`.

- **Data acquisition**

- Perform a multiplexed in situ HCR experiment (Choi *et al.*, 2016) and save the multi-channel image as a `.tif` file.

- **Data Normalization**

- Run the script by starting Matlab and typing `readoutreadin` at the prompt.
- The script will prompt you for the following information (see screenshot of Figure 2A):
 - * *image file name*: for example `zfish.tif`
 - * *number of analysis channels to be used for read-out/read-in*: must be less than or equal to the number of channels in the image file
 - * *index for each analysis channel*: allows the user to set each analysis channel to be any channel in the image file
 - * *label for each analysis channel*: used to label figures (for example, the name of the target mRNA for each channel)
 - * *maximum intensity for each analysis channel*: used for data normalization; for a given analysis channel, use the same value across all images in a study to enable quantitative comparisons between images (see Section S1.4.3 and Table S3 of Trivedi *et al.* (2018))
 - * *mean background intensity for each analysis channel*: used for data normalization; for a given analysis channel, use the same value across all images in a study to enable quantitative comparisons between images (see Section S1.4.3 and Table S3 of Trivedi *et al.* (2018))
 - * *voxel size*: number of pixels per voxel in x, y, z (see Table S2 of Trivedi *et al.* (2018))
- The script will display raw and normalized images:
 - * *Matlab Figure 1: raw image*. Depicts signal + background for pixels in each analysis channel plus a merge of all analysis channels (see screenshot of Figure 2B).
Saved as: `zfish-01-rawimage.pdf`
 - * *Matlab Figure 2: normalized image*. Depicts normalized signal estimate for voxels in each analysis channel plus a merge of all analysis channels (see screenshot of Figure 2B).
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- **Read-out from anatomical space to expression space**

- *Select anatomical regions of interest.*
 - * The script will prompt you for (see screenshot of Figure 3A):
 - *Number of regions of interest*: for example, 3 regions of interest
 - *Size of the regions of interest*: for example, 60×100 pixels (width \times height)
 - * The script will display a magnified merge view to assist with selecting regions of interest:
 - *Matlab Figure 3: anatomical regions of interest*. Use the cursor to define each rectangular region of interest in the magnified merge view (see screenshot of Figure 3B). Each voxel can be selected in at most one region.
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– *Plot pairwise expression scatter plots.* The script will then plot pairwise expression scatter plots using the voxel intensities in each region of interest:

* *Matlab Figure 4: raw expression scatter plots.* Raw voxel intensities for each pairwise combination of analysis channels. Voxel intensities are shaded by anatomical region of interest. Background and maximum values used for normalization are depicted with dashed lines (see screenshot of Figure 3C).

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* *Matlab Figure 5: normalized expression scatter plots.* Normalized voxel intensities for each pairwise combination of analysis channels. Voxel intensities are shaded by anatomical region of interest (see screenshot of Figure 3C).

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• Read-in from expression space to anatomical space

– *Select expression clusters of interest.*

* The script will prompt you for (see screenshot of Figure 4A):

· *Scatter plot number.* For example, the 4th pairwise scatter plot in Figure 5.

· *Number of expression clusters of interest.* For example, 3 expression clusters.

* The script will display a magnified view of the specified scatter plot to assist with selecting expression clusters of interest:

· *Matlab Figure 6: expression clusters of interest (voxels shaded by anatomical region).* Use the cursor to define a polygonal boundary for each expression cluster of interest (see screenshot of Figure 4B). Each voxel can be selected in at most one expression cluster. Voxels are shaded by anatomical region (see screenshot of Figure 4C).

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· *Matlab Figure 7: expression clusters of interest (voxels shaded by expression cluster).* The same expression clusters are displayed with all voxels shaded by expression cluster. Voxels that are not selected are displayed with a separate color (see screenshot of Figure 4C).

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– *Display segmented images.* The script will then plot images segmented by expression cluster:

* *Matlab Figure 8# series: segmented images.* For each region of interest, voxels are displayed segmented by expression cluster (columns) for each channel (rows; see screenshot of Figure 4D). An extra column on the left depicts *all* voxels and an extra column on the right depicts voxels *not selected* for an expression cluster. One figure is generated for each of the anatomical regions of interest (for example: *Matlab Figures 81, 82, 83* for 3 anatomical regions of interest).

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`zfish-08-readin-region1.pdf`

`zfish-08-readin-region2.pdf`

`zfish-08-readin-region3.pdf`

• Subcircuit expression scatter plots

– *Define subcircuits.* The script will prompt you for:

* *Number of subcircuits:* for example, 2 subcircuits (see screenshot of Figure 5A). *Note:* enter 0 to skip subcircuit analysis.

* *Definition of each subcircuit:* Each subcircuit is specified as a set of analysis channel numbers (see screenshot of Figure 5B). For image voxel i , the amplitude for a subcircuit of N target mRNAs is defined by

$$\sqrt{\left[(x_i^{target1})^2 + \dots + (x_i^{targetN})^2 \right]} / N \in [0, 1],$$

where $x_i^{target1}$ represents the normalized signal estimate for voxel i (see Section S1.4.3 of Trivedi *et al.* (2018)).

- The script will plot expression scatter plots for each pairwise combination of subcircuit axes:
 - * *Matlab Figure 9: subcircuit expression scatter plots (voxels shaded by anatomical region)* (see Figure 5C of Trivedi *et al.* (2018) and the screenshot of Figure 5C)
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 - * *Matlab Figure 10: subcircuit expression scatter plots (voxels shaded by expression cluster)* (see screenshot of Figure 5C)
Saved as: `zfish-10-subcircuits-bycluster.pdf`

If desired, the Matlab script (`readoutreadin.m`) can be customized to perform iterative read-out/read-in analyses in which the selection of anatomical regions and expression clusters is refined over the course of multiple trips back and forth between anatomical space and expression space.

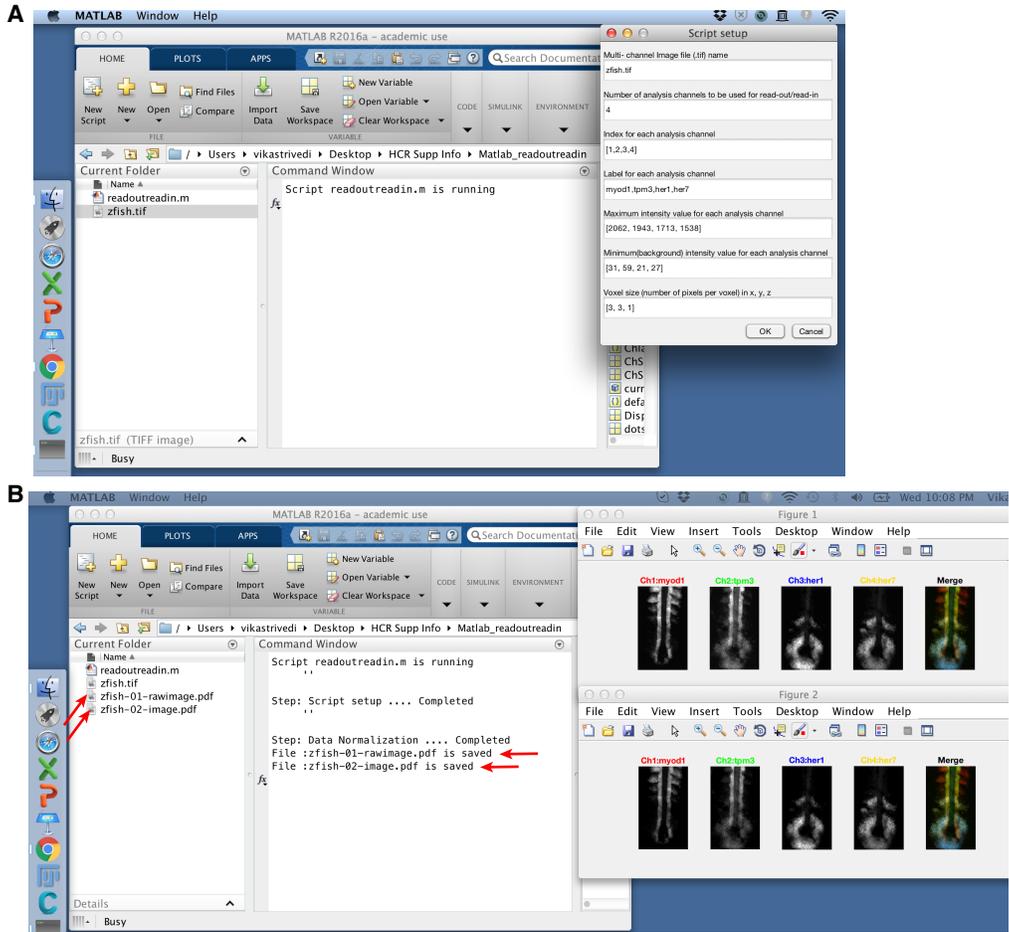


Figure 2. Data normalization. (A) Input normalization information. (B) Raw image (Matlab Figure 1) and normalized image (Matlab Figure 2).

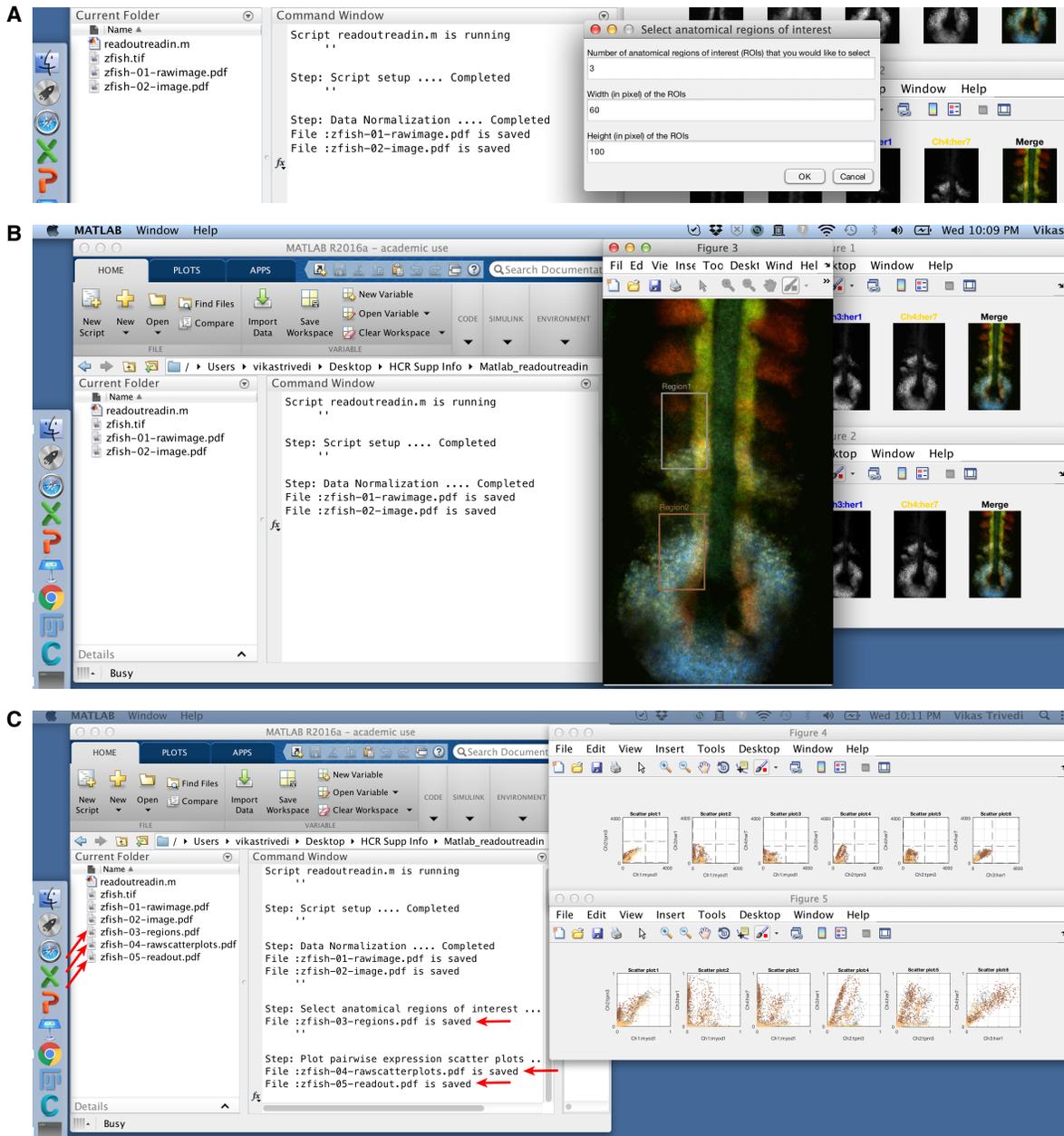


Figure 3. Read-out from anatomical space to expression space. (A) Input read-out information. (B) Select anatomical regions of interest (Matlab Figure 3). (C) Raw expression scatter plots (Matlab Figure 4) and normalized expression scatter plots (Matlab Figure 5).

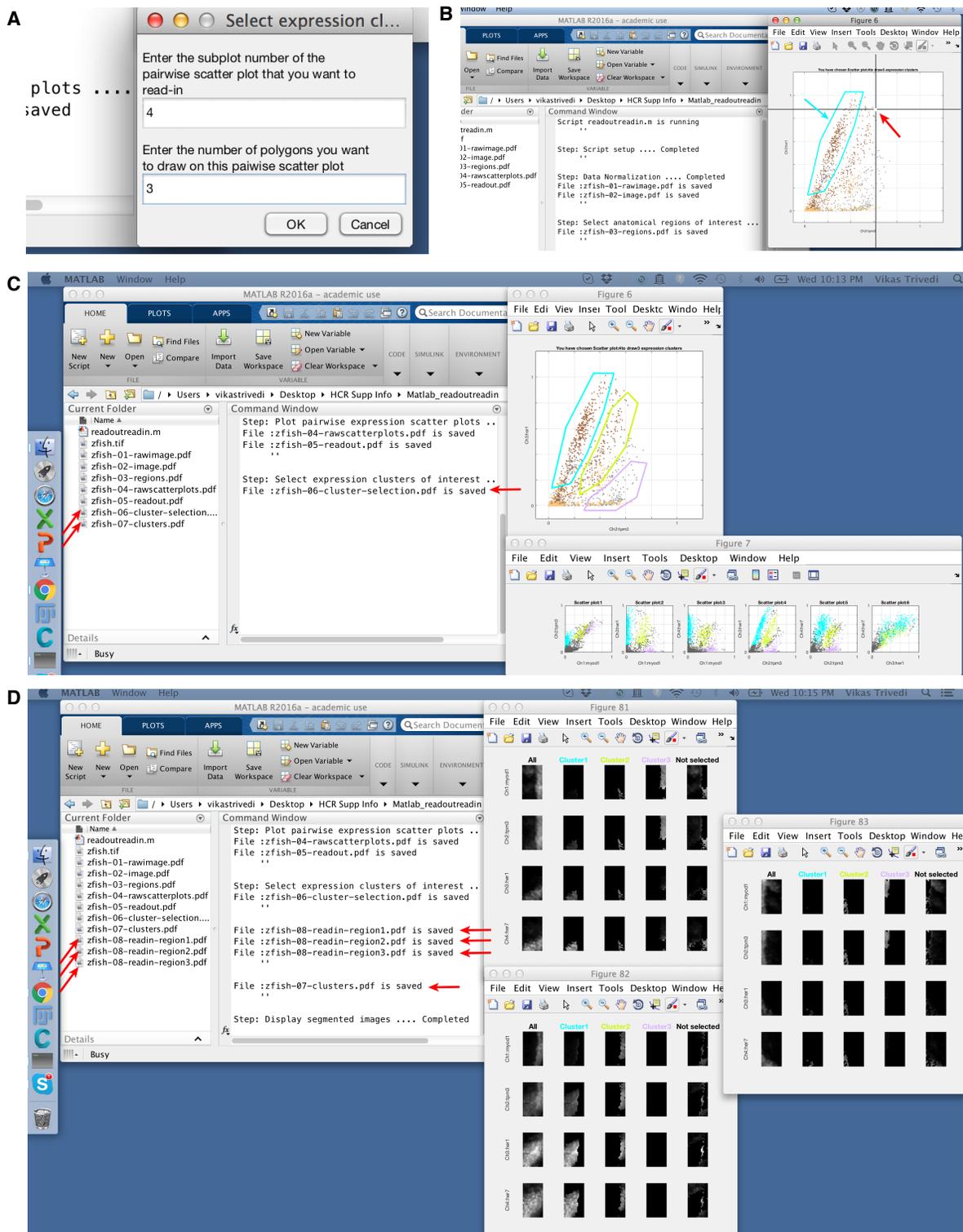


Figure 4. Read-in from expression space to anatomical space. (A) Input read-in information. (B) Select expression clusters of interest using the cursor to define a polygonal boundary for each expression cluster (Matlab Figure 6). (C) Expression clusters of interest with voxels shaded by expression cluster (Matlab Figure 7). (D) Segmented image (Matlab Figures 81, 82, 83 for 3 anatomical regions of interest).

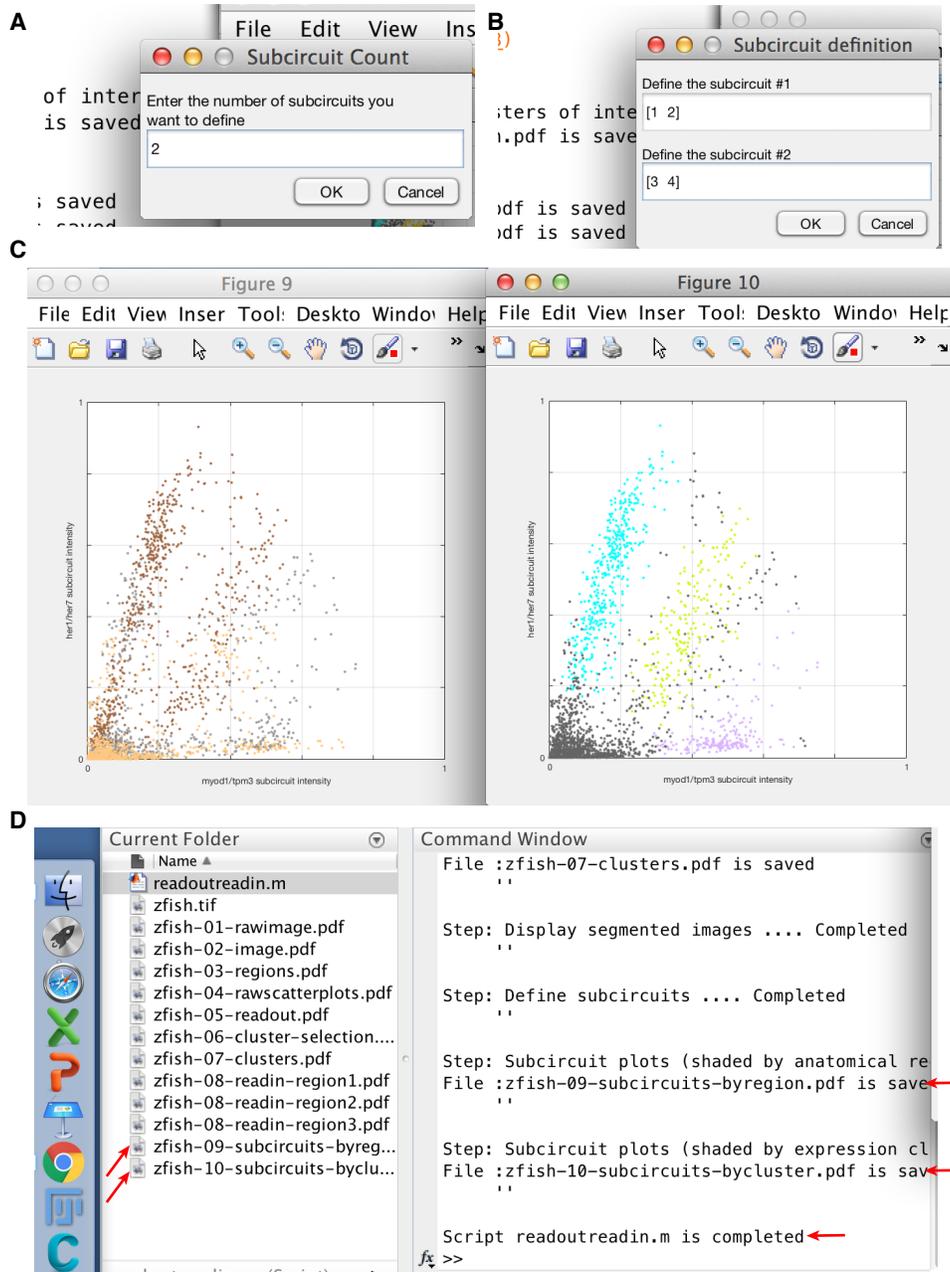


Figure 5. Subcircuit expression scatter plots. (A) Input number of subcircuits. (B) Define each subcircuit. (C) Subcircuit expression scatter plots with voxels shaded by anatomical region (Matlab Figure 9) or voxels shaded by expression cluster (Matlab Figure 10). (D) Output files saved.

References

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