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Title: SCAnED—An Open-Source Skin Segmentation Macro for Semi-Automated Cell and Nuclei Detection in Epidermal and Dermal Skin Compartments

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 22
Number of Shots: 32 (31 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Karin Pfisterer:** In our work we investigate regulatory mechanisms that maintain tissue homeostasis at molecular, cellular, structural and mechanical levels. Our goal is to understand pathological conditions, such as skin inflammation or cancer.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

1.1. **Karin Pfisterer:** With new high-resolution imaging and multi-omics tools, we can now map cellular and gene expression changes in tissues in 3D. This is really pushing our understanding of how diseases develop and progress.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What are the current experimental challenges?

1.2. **Karin Pfisterer:** One of the big challenges right now is the massive amount of data generated by advanced microscopy. And while there are more tools available to analyze images, they're often not user-friendly for people without a lot of experience.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.4.1*

What advantage does your protocol offer compared to other techniques?

1.3. **Parvaneh Balsini:** Our protocol offers a free, user-friendly tool for analyzing human skin cells in both epidermis and dermis. It improves accuracy over general tools and doesn't require coding or prior image analysis experience.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.1*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Image Analysis Using the SCAnED Macro

Demonstrators: Parvaneh Balsini and Karin Pfisterer

2.1. To begin, open the immunofluorescence images in Fiji or ImageJ using Bio-Formats with the default settings **[1]**. Do not split the channels during this step **[2]**.

2.1.1. WIDE: Talent taking a seat at the computer table and opening the image. **Author's NOTE**: Show the talent taking a seat only and do not show the opening of the image on the computer

2.1.2. SCREEN: 68746_SCREEN_2.1.2.mp4

2.2. Download the SCAnED *(scanned)* macro and run it by selecting **Plugins**, then **Macros**, and clicking **Run [1]**. Select the SCAnED macro script file when prompted **[2]**.

2.2.1. SCREEN: 68746_SCREEN_2.2.1.mp4

2.2.2. SCREEN: 68746_SCREEN_2.2.2.mp4

2.3. Follow the on-screen instructions provided by the macro **[1]**.

2.3.1. SCREEN: 68746_SCREEN_2.3.1.mp4

2.4. Select the **E-cadherin** channel when prompted by the macro **[1]**.

2.4.1. SCREEN: 68746_SCREEN_2.4.1.mp4

2.5. Next, navigate to **Image**, choose **Adjust**, then **Threshold** to manually adjust the threshold for the E-cadherin channel **[1]**. Click **OK** once the epidermal region is fully captured **[2]**.

2.5.1. SCREEN: 68746_SCREEN_2.5.1.mp4 00:01-00:12

2.5.2. SCREEN: 68746_SCREEN_2.5.2.mp4

2.6. Then, select the **DAPI** *(dapee)* channel when prompted to proceed with segmentation **[1]**.

2.6.1. SCREEN: 68746_SCREEN_2.6.1.mp4 00:00-00:10

3. Nucleus and Cell Segmentation

Demonstrator: Parvaneh Balsini

3.1. Auto-define the intensity levels for nuclei segmentation when using the **threshold** function **[1]**.

3.1.1. SCREEN: 68746_SCREEN_3.1.1.mp4 00:00-00:07

3.2. For StarDist *(star-dist)*, open the plugin in ImageJ **[1]**.

3.2.1. SCREEN: 68746_SCREEN_3.2.1.mp4 00:07-00:14

3.3. Load the nuclei detection model into the StarDist graphical interface **[1]** and click on the nuclei channel to select it for analysis **[2]**.

3.3.1. SCREEN: 68746_SCREEN_3.3.1.mp4 00:12-00:20

3.3.2. SCREEN: 68746_SCREEN_3.3.2.mp4 00:04-00:12

3.4. Now, adjust the tile settings in StarDist to match the expected patch size of 256 by 256 pixels **[1]**.

3.4.1. SCREEN: 68746_SCREEN_3.4.1.mp4

3.5. When prompted, select **Yes** to measure marker intensity within the nuclei **[1]**.

3.5.1. SCREEN: 68746_SCREEN_3.5.1.mp4 00:01-00:10

3.6. Then, repeat the segmentation and analysis process for each fluorescence channel **[1]**.

3.6.1. SCREEN: 68746_SCREEN_3.6.1.mp4

3.7. After all channels are processed, save the output data as comma-separated value files as instructed by the macro **[1]**.

3.7.1. SCREEN: 68746_SCREEN_3.7.1.mp4 00:00-00:07

3.8. For cytoplasmic region, when nuclei segmentation has been performed using the threshold function, apply binary dilation to expand the nuclear region of interest **[1]**.

3.8.1. SCREEN: 68746_SCREEN_3.8.1.mp4

3.9. When StarDist has been used for nuclei segmentation, apply enlargement expansion to the nuclear region of interest **[1]**.

3.9.1. SCREEN: 68746_SCREEN_3.9.1.mp4 00:00-00:08 and 00:22-00:25

3.10. Select **Yes** when prompted to measure marker intensity within the whole cell region **[1]**.

3.10.1. SCREEN: 68746_SCREEN_3.10.1.mp4 00:00-00:09

3.11. Then, save the resulting data files as comma-separated value format according to macro instructions **[1]**.

3.11.1. SCREEN: 68746_SCREEN_3.11.1.mp4

3.12. Establish marker-specific intensity thresholds using isotype control samples **[1]**.

3.12.1. SCREEN: 68746_SCREEN_3.12.1.mp4 00:20-00:27

4. Classification and Visualization of Extracted Single-Cell Data

Demonstrators: Parvaneh Balsini and Karin Pfisterer

4.1. Download the appropriate Jupyter *(Jupiter)* Notebook **[1]** and navigate to Google Colab **[2]** to select **File** followed by **Open notebook**, choose the file **[3]**, and run the code cells sequentially **[4]**. When prompted with the **Choose Files** option, click the button and upload the comma-separated value file **[5]**.

4.1.1. SCREEN: 68746_SCREEN_4.1.1.mp4 *Video editor: Please speed up*

4.1.2. SCREEN: 68746_SCREEN_4.1.2.mp4 00:05-00:10

4.1.3. SCREEN: 68746_SCREEN_4.1.3.mp4 00:01-00:12

4.1.4. SCREEN: 68746_SCREEN_4.1.3.mp4 00:20-00:26

4.1.5. SCREEN: 68746_SCREEN_4.1.4.mp4

4.2. For each antibody, calculate the mean fluorescence intensity using the isotype control files **[1]**. To determine the cut-off thresholds, open the DotPlot notebook, upload the

isotype control data, and run the code **[2]**.

4.2.1. SCREEN: 68746_SCREEN_4.2.1.mp4

4.2.2. SCREEN: 68746_SCREEN_4.2.2.mp4 *Video editor: Please speed up*

4.3. Based on the distribution of dots in the plots for both isotype control and specific antibody data, select an intensity threshold to distinguish background signal from true marker expression **[1]**. Use this threshold to classify each cell as positive or negative for the given marker **[2]**.

4.3.1. SCREEN: 68746_SCREEN_4.3.1.mp4

4.3.2. SCREEN: 68746_SCREEN_4.3.2.mp4 00:05-00:18

4.4. Finally, create dot plots to visualize co-expression patterns of markers by running the DotPlot code in the Google Colab notebook **[1]**.

4.4.1. SCREEN: 68746_SCREEN_4.4.1.mp4 00:00-00:04 and 01:07-01:12

Results

5. Results

5.1. In manually segmented images, Vimentin-positive cells showed a strong cytoplasmic signal with clear ROI boundaries covering the full cell body **[1]**.

5.1.1. LAB MEDIA: Figure 11. *Video editor: Highlight A image*.

5.2. The SCAnED semi-automated segmentation captured VIM-positive cells with similar boundaries using a threshold and dilation approach **[1]**.

5.2.1. LAB MEDIA: Figure 11. *Video editor: Highlight image B* .

5.3. Quantitative comparison of vimentin intensity in 21 cells showed no significant difference between manual and SCAnED segmentation **[1]**.

5.3.1. LAB MEDIA: Figure 11. *Video editor: Highlight the two bars in C*

5.4. Histogram analysis revealed a higher frequency of Vimentin-positive cells in psoriasis dermis **[1]** compared to healthy skin **[2]**.

5.4.1. LAB MEDIA: Figure 8. *Video editor: Highlight the red bars for "P".*

5.4.2. LAB MEDIA: Figure 8. *Video editor: Highlight the blue bars for "H"*

5.5. Dot plot analysis of dermal cells showed increased CD90+ *(C-D-ninety-positive)* VIM+ *(vimentin positive)* cells in psoriasis **[1]** compared to healthy skin **[2]**.

5.5.1. LAB MEDIA: Figure 9A and 9B. *Video editor: Highlight the green dot cluster (double-positive cells) in the psoriasis plot (B)*

5.5.2. LAB MEDIA: Figure 9A and 9B. *Video editor: Highlight the green dot cluster (double-positive cells) in the in the healthy plot (A)*.

5.6. In the epidermis, psoriasis samples showed an increase in CD90-positive vimentin-negative cells, possibly immune cells, compared to healthy skin **[1]**.

5.6.1. LAB MEDIA: Figure 9C,D. *Video editor: Highlight the red dot cluster in D*

5.6.2. LAB MEDIA: Figure 9C,D. *Video editor: Highlight the red dot cluster in C*

5.7. Vimentin-positive cell counts in psoriatic and healthy epidermis were comparable

between SCAnED and QuPath *(Q-Path)* **[1]**.

5.7.1. LAB MEDIA: Figure 13F. *Video editor: Highlight the bars representing both healthy and psoriasis groups*.

5.8. Visual segmentation showed SCAnED more accurately assigned border cells to the epidermis than QuPath **[1]**, which misassigned several **[2]**.

5.8.1. LAB MEDIA: Figure 13A and B. *Video editor: Highlight A*.

5.8.2. LAB MEDIA: Figure 13A and B. *Video editor: Highlight B*.

1. **Immunofluorescence**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/immunofluorescence
   IPA: /ɪˌmjuːnoʊfloʊˈrɛsəns/
   Phonetic Spelling: ih-myoo-noh-flu-reh-sens
2. **Bio-Formats**
   Pronunciation link:
   No confirmed link found
   IPA: /ˌbaɪ.oʊ-ˈfɔr.mæts/
   Phonetic Spelling: bye-oh-for-mats
3. **SCAnED**
   Pronunciation link:
   No confirmed link found
   IPA: /ˈskænd/
   Phonetic Spelling: skand
4. **E-cadherin**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/ecadherin
   IPA: /ˌiːˈkædərɪn/
   Phonetic Spelling: ee-kad-eh-rin
5. **DAPI**
   Pronunciation link:
   No confirmed link found
   IPA: /ˈdeɪpiː/
   Phonetic Spelling: day-pee
6. **StarDist**
   Pronunciation link:
   No confirmed link found
   IPA: /ˈstɑːr-dɪst/
   Phonetic Spelling: star-dist

7. **QuPath**
   Pronunciation link:
   No confirmed link found
   IPA: /ˈkjuːˌpæθ/
   Phonetic Spelling: cue-path
8. **Isotype**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/isotype
   IPA: /ˈaɪsoʊtaɪp/
   Phonetic Spelling: eye-so-type