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Scriptwriter Name: Swati Madhu
Supervisor Name: Anastasia Gomez
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Title: Artificial Intelligence Approaches to Assessing Primary Cilia

Authors and Affiliations:

Ruchi Bansal[1], Staci E. Engle[1], Tisianna K. Kamba[1], Kathryn M. Brewer[1], Wesley R. Lewis[2], Nicolas F. Berbari[1, 3, 4]

[1]Department of Biology, Indiana University-Purdue University Indianapolis
[2]Nikon Instruments Inc.
[3]Stark Neurosciences Research Institute, Indiana University
[4]Center for Diabetes and Metabolic Diseases, Indiana University School of Medicine

Corresponding Authors:

Nicolas F. Berbari nberbari@iupui.edu

Email Addresses for All Authors:

bansalr@iu.edu
stengle@iu.edu
tkamba@iu.edu
katmbrew@iu.edu
wesley.lewis@nikon.com
nberbari@iupui.edu

Author Questionnaire

1. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interview Statements are read by JoVE's voiceover talent.

Current Protocol Length

Number of Steps: 19
Number of Shots: 28

Introduction

1. Introductory Interview Statements

1.1. Current methods of cilia analysis are labor-intensive and prone to error and bias. Our approach seeks to streamline time and effort while mitigating potential errors.

1.1.1. *2.3.1*

1.2. The main advantage that this technique offers is increased rigor and reproducibility in quantitative image analysis.

1.2.1. *2.4.1 and 2.4.2*

1.3. This type of approach is not only relevant to cilia analysis but can be broadly applied to many cell biological questions, including those dealing with other organelles and cytoskeletal proteins.

1.3.1. *4.3.1*

Protocol

2. Artificial Intelligence Training to Identify the Cilia

2.1. To begin, open the training dataset, select **File** from the menu, click **Import/Export** and select **Create ND** *(N-D)* **File from File Sequence [1].** Select the folder containing the training dataset, and the list of files will open in the center of the dialogue window **[2]**.

2.1.1. LAB MEDIA: Video 62521_R0: 00:35 to 00:47

2.1.2. LAB MEDIA: Video 62521_R0: 00:48 to 00:56

2.2. Manually define the organization of files using at least one option in the drop-down menu. Enter the corresponding numerical values under each selected option and select **None** wherever options are not selected **[1]**. Click **Convert** to open the ND document **[2]**.

2.2.1. LAB MEDIA: Video 62521_R0: 01:03 to 01:05 and 01:13 to 01:25

2.2.2. LAB MEDIA: Video 62521_R0: 01:40 to 01:49 *Video editor: Speed up the video*

2.3. To calibrate the image, right-click on the **Uncalibrated** option in the lower left-hand corner of the Image, click **Calibrate Document**, then click **Pixel Size,** enter the value, and click **OK [1]**.

2.3.1. LAB MEDIA: Video 62521_R0: 01:57 to 02:15 *Video Editor: Speed up the video.*

2.4. Select **View**, **Analysis Controls,** open the **Binary Toolbar,** and select **AutoDetect** or **Draw Object [1]** to hand-identify cilia by precisely tracing individual ciliary structures on all the opened frames **[2]**.

2.4.1. LAB MEDIA: Video 62521_R0: 02:23 to 02:32 and 02:44 to 02:48

2.4.2. LAB MEDIA: Video 62521_R0: 03:02 to 03:11

2.5. For training the Ai *(A-I)*, select **NIS.ai** *(N-I-S-dot-A-I)*, click **Train Segment.ai** *(Segment-dot-A-I)* to open the Train Segment.ai box, and then select the source channel to be used for training **[1]**. Select the appropriate ground truth binaries to train the Ai **[2]**.

2.5.1. LAB MEDIA: Video 62521_R0: 04:09 to 04:20

2.5.2. LAB MEDIA: Video 62521_R0: 04:35 to 04:39 and 04:49 to 04:52

2.6. Select the required number of iterations to train the Ai depending on the binaries' size and distribution. Then, select the destination folder to save the trained Ai file **[1]** and click **Train** to train the software. This process takes several hours **[2]**.

2.6.1. LAB MEDIA: Video 62521_R0: 04:49 to 05:02 *Video editor: Speed up the video*

2.6.2. LAB MEDIA: Video 62521_R0: 05:17 to 05:21; 05:33 to 05:35 and 05:39 to 05:41

3. Identify cilia using trained Ai

3.1. Open the experimental confocal images of cilia as described before by converting the sample .tif *(dot-T-I-F)* files to .nd2 *(dot-N-D-2)* files **[1]**.

3.1.1. LAB MEDIA: Video 62521_R0: 06:22 to 06:33

3.2. In the pop-up window select **Multipoint** from the first drop-down menu and enter a value corresponding to the total number of the images **[1]**.

3.2.1. LAB MEDIA: Video 62521_R0: 06:54 to 07:03

3.3. In the second drop-down box, select **Wavelength** and change the value to the total number of the channels in the folder. The software will automatically unlock a **Wavelength** selection window located at the bottom right end of the pop-up window **[1]**.

3.3.1. LAB MEDIA: Video 62521_R0: 07:07 to 07:15

3.4. In the **Wavelength** selection window, use the **Color** drop-down menu to select the color of each channel. Provide each channel with a different name under the *Name* column. Once all information is updated, click **Convert [1]**.

3.4.1. LAB MEDIA: Video 62521_R0: 07:28 to 07:59 *Video editor: Speed up the video*

3.5. Calibrate the images as described before. Make sure the pixel size of the experimental dataset is consistent with that of the training dataset **[1]**.

3.5.1. LAB MEDIA: Video 62521_R0: 08:15 to 08:22

3.6. Identify cilia on the first channel by using the trained Ai from the previous step. Open **NIS.ai** from the menu, select **Segment.ai**, then select **ACIII** *(A-C-three)* in the **Source channels [1].** Then identify the cilia on the second channel by selecting **MCHR1** *(M-C-*

H-R-one) in the **Source channels.** The software will draw binaries on the labeled cilia **[2]**.

3.6.1. LAB MEDIA: Video 62521_R0: 08:30 to 08:42 and 08:52 to 09:03 *Video editor: Speed up the video*

3.6.2. LAB MEDIA: Video 62521_R0: 09:10 to 09:25 *Video editor: Speed up the video*

3.7. Next, check the images for any misidentified binaries. Select **Delete Object** in the **Binary Toolbar** to manually delete the misidentified binaries **[1]**.

3.7.1. LAB MEDIA: Video 62521_R0: 09:36 to 09:44

4. Cilia Length and Intensity Measurement

4.1. Once the cilia have been identified and segmented, analyze different cilia parameters such as lengths and intensities using the general analysis-3 tool. Select **Image** from the menu and click **New GA3** *(G-A-3)* **Recipe.** A new window with a blank space in the center will be opened **[1].**

4.1.1. LAB MEDIA: Video 62521_R0: 10:17 to 10:24

4.2. GA3 will automatically detect the binaries appropriately labeled according to the Ai and include the corresponding node **[1]**. GA3 will also automatically detect the channels in the images and display their tabs under **Channels [2].**

4.2.1. LAB MEDIA: 62521_screenshot_1: 00:07 to 00:14

4.2.2. LAB MEDIA: 62521_screenshot_1: 00:18 to 00:22

4.3. The Ai will segment all cilia-like objects in the frame and detect incomplete cilia along the edges of the frame. To remove them, select **Binary processing**, **Remove objects** and then drag the **Touching Borders** node into the blank space, and Connect the node to the appropriate binaries. **[1]**.

4.3.1. LAB MEDIA: Video 62521_R0: 11:23 to 11:51

4.4. To measure cilia length, select **Measurement, Object Size,** and then **Length**. Drag and drop the parameter to the center and connect to the appropriate binary node **[1]**. To measure cilia intensities, select **Sum Obj Intensity** *(sum object intensity)*. Drag and drop the parameter to the center and connect to the appropriate binary node and channel of interest **[2]**.

4.4.1. LAB MEDIA: Video 62521_R0: 12:09 to 12:24 *Video editor: Speed up the video.*

4.4.2. LAB MEDIA: Video 62521_R0: 12:33 to 12:44

5. Colocalization Studies

5.1. In the **Measurement** menu, go to **Object Ratiometry** and select **Manders Coefficient** to set up the colocalization pathway in GA3 by measuring the overlap of two channels within individual cilia. Drag and drop the Manders Coefficient node in the blank space and connect it to the appropriate binary and channels **[1]**.

5.1.1. LAB MEDIA: Video 62521_R0: 12:50 to 13:20 *Video editor: Speed up the video*

5.2. Append the measurements in a single table by opening the **Data Management** menu**.** In the **Basic** category, select **Append Column [1]** and then click **Run Now** to measure cilia. All the measurements will appear in a single output table **[2].**

5.2.1. LAB MEDIA: Video 62521_R0: 13:35 to 13:55

5.2.2. LAB MEDIA: Video 62521_R0: 14:02 to 14:09

Results

6. Results: Cilia Measurement Using Ai

6.1. The representative images show **[1]** that the trained Ai properly identified cilia *in-vitro* in the images of IMCD cells, primary hypothalamic cultures, and hippocampal cultures **[2]** but no other non-ciliary structures such as cytokinetic bridges **[3]**.

6.1.1. LAB MEDIA: Figure 2

6.1.2. LAB MEDIA: Figure 2 *Video editor: Please emphasize purple cilia in binary mask images and corresponding green cilia in Ac. Tub. And ACIII images on the left.*

6.1.3. LAB MEDIA: Figure 2A *Video editor: Please emphasize the asterisk labeled green structure in Ac. Tub. Image.*

6.2. The length of the cilia ranged from 0.5 to 4.5 micrometers in IMCD cells **[1]**, and 2 to 12 micrometers in the hypothalamic and hippocampal culture **[2]**.

6.2.1. LAB MEDIA: Figure 2A *Video editor: Please emphasize the graph.*

6.2.2. LAB MEDIA: Figure 2B and C *Video editor: Please emphasize the graph.*

6.3. The Ai measured the lengths of AC-3 labeled cilia *in-vivo* **[1]** in the images of arcuate nucleus, paraventricular nucleus, and cornu ammonis-1 regions **[2]**.

6.3.1. LAB MEDIA: Figure 3

6.3.2. LAB MEDIA: Figure 3A and B *Video editor: Please emphasize the green-colored cilia in the ACIII image row and purple cilia in the Binary mask image raw.*

6.4. According to the analysis, hypothalamic cilia *in-vivo* ranged from 1 to 15 micrometers **[1]**, while cilia in the cornu ammonis region ranged from 1 to 10 micrometers **[2]**.

6.4.1. LAB MEDIA: Figure 3C *Video editor: Please emphasize black and brown bars.*

6.4.2. LAB MEDIA: Figure 3C *Video editor: Please emphasize gray bars.*

6.5. Interestingly, the intensity of the ciliary MCHR1 **[1]** was stronger in the paraventricular nucleus than that in the arcuate nucleus **[2]**.

6.5.1. LAB MEDIA: Figure 4

6.5.2. LAB MEDIA: Figure 4A and B *Video editor: Please emphasize the PVN-Binary mask image in Figure 4A and gray dots of the PVN bar in Figure 4B.*

6.6. The intensities of MCHR1 against AC-3 were plotted to measure their overlap **[1]**. The majority of cilia were positive for both markers **[2]**, while some cilia were positive for either AC-3 or MCHR1 **[3]**.

6.6.1. LAB MEDIA: Figure 5

6.6.2. LAB MEDIA: Figure 5A, B, and D *Video editor: Please emphasize the merge images in Figure 5A and Figure 5B.*

6.6.3. LAB MEDIA: Figure 5A, B, and D *Video editor: Please, emphasize the scattered gray and black dots in the graph of Figure 5D.*

6.7. To quantify colocalization of MCHR1 within ACIII, Mander's overlap coefficient was measured **[1]**, and there was a significant increase in the overlap in the paraventricular nucleus than in the arcuate nucleus **[2]**.

6.7.1. LAB MEDIA: Figure 5C

6.7.2. LAB MEDIA: Figure 5C *Video editor: Please emphasize PVN bar.*

6.8. To measure intensity along the length of the cilia, cilia polarity was defined using Centrin-2-GFP as the basal body marker **[1]**. This allowed to distinguish the base of cilia from the tips of ARL13B-mCherry *(A-R-L-13-B-m-cherry)* positive cilia **[2]**.

6.8.1. LAB MEDIA: Figure 6

6.8.2. LAB MEDIA: Figure 6A

6.9. Changes in ARL13B intensity along the length of cilia were observed **[1]**, where ARL13B intensity was higher at the base than at the tip of the cilium **[2]**.

6.9.1. LAB MEDIA: Figure 6B

6.9.2. LAB MEDIA: Figure 6C *Video editor: Please emphasize the proximal and distal bars in both graphs.*

Conclusion

7. Conclusion Interview Statements

7.1. When analyzing data with this approach, it is important to make sure the quality and resolution of the experimental dataset is consistent with that used to train Ai.

7.1.1. *3.1.1*

7.2. The main utility of this approach is after detecting cilia by Ai, the user can be creative about which properties are analyzed by customizing the analysis workflow integrated within the software.

7.2.1. *4.4.1*