VOID WHIZZARD

Free open-source software for high-throughput quantification of the spontaneous void spot assay

USER GUIDE

VERSION 1.4

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VOID WHIZZARD DEVELOPMENT TEAM/ FUNDING

Development Team

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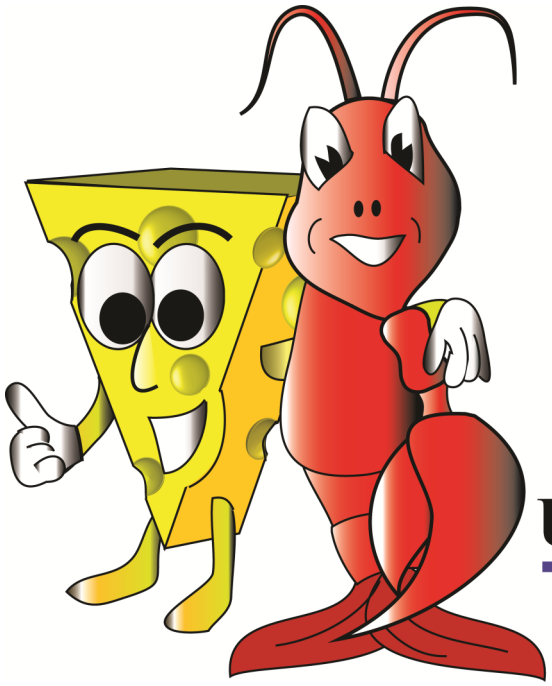
This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.



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WHY USE VOID WHIZZARD?

Several methods currently exist for quantification of urinary function in mice. The spontaneous void spot assay (a.k.a VSA, Void Spot on Paper Assay, VSOP) has numerous advantages over alternatives. Requiring only a standard rodent housing cage and filter paper, the assay is extremely cost effective and requires no access to specialized equipment. The non-invasive nature of the assay allows for multiple tests to be performed throughout an animal's life, and also permits pre and post treatment experimental design, making it the premier assay for evaluating urinary function over time.

A drawback of the VSA is the time required for analysis of the filter papers after the assay has been conducted. Also, critics of the assay claim that analysis is subjective and results can vary from user to user. This software package addresses these concerns by simplifying and automating the analysis process. Automated measurement drastically reduces time for analysis, whether users quantify filter papers individually or in large batches. Further, Void Whizzard applies consistent quantification techniques (via standardized algorithms) across images, eliminating user bias. Void Whizzard is designed for efficient high-throughput analysis of VSA filter papers while retaining flexibility: the software allows user input at discrete steps to accommodate the potential for differing experimental and analytical parameters. Users can test filter papers in large batches, drastically reducing time for analysis.

We provide Void Whizzard at no cost as an extension of FIJI, a redistribution of ImageJ designed for the life sciences by researchers at the UW Madison Laboratory of Optical and Computational Instrumentation (LOCI). All that we ask is that you cite the software in any resulting publications (for citation instructions see page 12)

Thank you,
Void Whizzard Development Team

VOID WHIZZARD at a glance:

- High-throughput analysis
- Flexible analysis parameters
- Objective measurements
- User-friendly interface
- Free and open source
- Part of FIJI plugin suite

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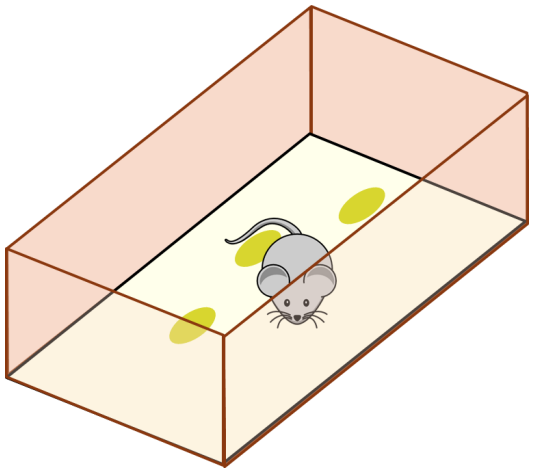
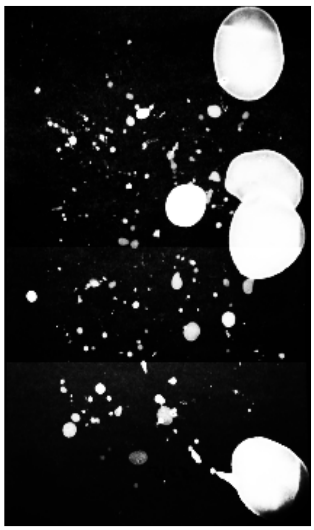
WELCOME!

Void Whizzard is a free plugin for FIJI, created to simplify and expedite quantification of the spontaneous void spot assay. It is designed to objectively quantify urinary function without sophisticated computer programming requirements.



QUICK START GUIDE

1) **Download FIJI**
   Software available for free at https://imagej.net/Fiji/Downloads
2) **Install Void Whizzard Plugins**
   In FIJI, subscribe to the Vezina Lab update site: http://sites.imagej.net/VezinaLab
3) **Run Void Whizzard**
   Follow on-screen instructions.
4) **Cite**
   Instructions for citations can be found on page 12.



Void Whizzard Analysis Results

1) Total number of urine spots
2) Total volume of urine
3) Area of urine spots
4) Spatial localization of voiding events
5) Distribution of spots into user-defined bins

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BEFORE YOU START

PLATFORMS SUPPORTED

- **Windows XP, Vista, 7, 8 and 10**
- **Mac OS X 10.8 "Mountain Lion" or later**
- **Linux on amd64 and x86 architectures**

SETUP AND INSTALLATION

Install Void Whizzard

Void Whizzard is comprised of three components:

1. FIJI open-source image processing software
2. Void Whizzard plugin
3. Cropping Tool plugin

To install automatically via FIJI:

1) Install FIJI

FIJI is an open-source image processing package. It is a redistribution of the popular ImageJ image processing software. FIJI was developed by contributors around the world, and funded from various sources. It is maintained by the Laboratory for Optical and Computational Instrumentation (LOCI) at the University of Wisconsin-Madison. FIJI can be installed using instructions at https://imagej.net/Fiji/Downloads.

2) Subscribe to Void Whizzard

Once FIJI is installed navigate to Help>>Update…. A dialog box will open. Press "Manage update sites." Another window will open. Click "Add update site." Double click the new row that was created to change the name to 'Void Whizzard.' Then double click the URL box and enter "http://sites.imagej.net/VezinaLab/". Click "Close." Click "Apply changes." Restart FIJI. The "Cropping tool" and "Void Whizzard" should now appear under the Plugins menu in FIJI.

USING VOID WHIZZARD

RUNNING THE VOID SPOT ASSAY

Detailed protocols for the suggested method of performing the spontaneous void spot assay can be found at http://www.urology.wisc.edu/research/u54-george-m-obrien-center-for-benign-urology-research/rodent-urinary-function-testing-ruft-core/services/#C1

Assay Reference:

Keil KP, Abler LL, Altmann HM, Bushman W, Marker PC, Li L, Ricke WA, Bjorling DE, Vezina CM. 2014. Influence of animal husbandry practices on void spot assay outcomes in C57BL/6J male mice. ***Neurourol Urodyn*** 2:192-198.

RUNNING VOID WHIZZARD

1) Create a standard curve file.

NOTE: The standard curve is an **optional** file containing data necessary to convert spot area to volume and is only required if users seek to translate void spot areas to urine volumes. If urine volume endpoints are not needed, skip to step 2.

This plugin creates a standard curve from a series of urine spots of known volumes. Urine spot area is affected by a variety of user parameters, including the filter paper type used in the void spot assay. Every user should therefore generate a standard curve to maximize accuracy of urine volume calculations. Only one standard curve file needs to be created per user and can be reused multiple times provided their is no change in the type of paper used in the void spot assay.

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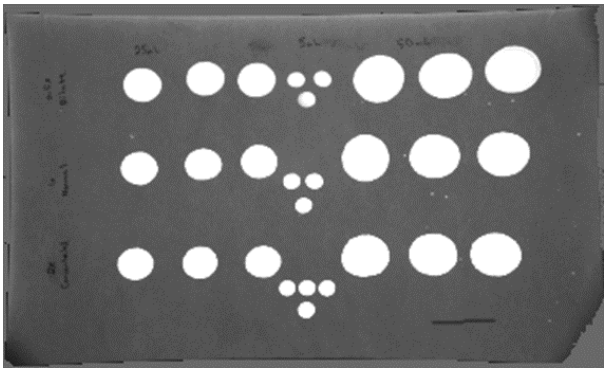
The volume to area relationship is calculated as follows:

A. An image like the one below is created using saline as the spotting medium. Pipet saline onto the filter paper and outline spots with pencil or pen before they dry. Saline spots in the image below are highlighted white to enhance visualization. You do not need to highlight them. Use a pencil or pen to create a line of known length on the paper.

Spots should be of a known volume and should not overlap. While at least two spots of different sizes are required to compute the relationship between volume and area, it is recommended that at least five different volumes are used and that each volume is spotted in triplicate to increase measurement accuracy.

The image to the right has a line of known length in the bottom right corner and features nine 25µL spots, ten 5µL spots, and nine 50µL spots.



B. Spotted filter papers should be imaged according to the user's standard operating procedure for imaging void spot assay filter papers. Save the image (.tif) and import into FIJI.

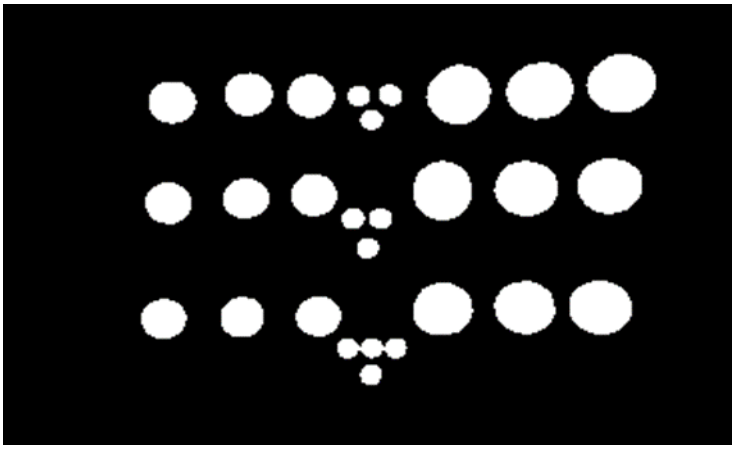
C. Change the fill color to white by navigating to Edit>>Options>>Colors…. Change the foreground color to white.

D. Use the “Freehand Selection” tool  to trace a spot and then navigate to Edit>>Fill… to fill the spot with white. Repeat for all spots. Following this, the image should look similar to the image above.

E. Use the “*Straight*” tool  to draw a line over the line of known length. Then open Analyze>>Set Scale…. The distance in pixels should be indicated when the dialog box opens. Enter the line length into the "known distance" field and enter the unit of measurement in the "Unit of length field. Select ok.

USING VOID WHIZZARD

F. Threshold the image to make urine spots appear white and the background appear black by navigating to Image>>Adjust>>Threshold…. and using the slider to adjust the threshold such that on the white spots are highlighted. Select 'Apply' to apply the threshold to your image. The image below is the threshold of the image from step A.



G. Use the 'Wand Tool' to click on an image and select it. Then press the "M" key to measure the spot. The area listed in the results window is calculated using the units of measurement provided in step E. Repeat for each spot.

H. The mean area and volume should be determined from the representative spots. Enter the area and volume for each spot in the conv.txt file as described below.

The conv.txt file contains two lines: the first line lists spot volumes, and the second line lists spot areas. The first and second lines are used to create the linear relationship between area and volume. Both lines consist of a set of values separated by a single space. Both lines must contain the same number of data points and must only contain numerical characters. Any other format will cause an error and terminate the macro. To generate the conv.txt file you can use any text editing software. In Microsoft word, use 'save as' to save the file as a Plain Text Format (.txt). The conv.txt file must be titled "conv.txt". Save the conv.txt file in the same folder as the VSA images to be processed.

```
10 20 30 40 50 60 70 80 90 100   <-- Volume
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1   <-- Area
```

2) Image VSA papers.

VSA papers should be imaged in landscape orientation and saved as .tif format files. For example, the image should be “VSAimage.tif” (important: tif should be spelled in all lowercase). Create a folder for exclusive storage of VSA image files and save image files to this directory. Create a separate output folder for cropped images, to be used in step 4. **NOTE:** Placement of non-VSA image or conv.txt files into this dedicated folder will cause errors.

3) Determine width of VSA paper in pixels.

Identify the pixel width of the VSA paper. Pixel width is a variable influenced by the type of camera used for imaging. To determine pixel width, open one of the VSA images in FIJI and use the “*Straight*” tool to measure the vertical distance between the top and bottom paper edges. Length is indicated in the (Fiji Is Just) ImageJ window as line is being drawn. Record the paper's computed pixel width which will remain constant for all subsequent images. This value will be utilized in step 4 below.

4) Run Cropping Tool Plugin.

The Cropping tool includes a "Straightener" function which will straighten each image, then crop it. This function renders each image at appropriate orientation and size for analysis using the Void Whizzard software. Select Plugins>>Cropping tool.... and into the dialog box, designate the directory which contains raw VSA images.

a) A second dialog box will open to select the output folder for cropped images.

b) The first image will open. Select the “*Straight*” tool. Place the cursor over the center of the left edge of the VSA paper and draw a line from that point to the approximate center of the right edge of the paper. Press OK in the dialog box that says “Press OK to continue.”

c) A new dialog box opens titled “Straightener” in which the title of the image appears. In the Line Width box, manually enter the pixel distance you measured in step 3 above. Press OK.

d) The first image closes and the next image opens. On this new image, draw another line from the center left edge to the center right edge. You do not need to select the line tool as it should be selected by default. The line width information entered in step 4c allows visualization of a transparent overlay box during line drawing. This overlay box can be used to aid in accurate tracing of the orientation and dimensions of the imaged VSA paper.

e) Press OK to continue and proceed through all of your images.

5) Start the Void Whizzard plugins.

Start the macro by clicking on Plugins>>Void Whizzard.... The macro will open an interface for custom adjustments to settings and outputs.

6) Establish user input settings.

A user settings window will open at the beginning of each new Void Whizzard session. There are several customizable options.

Spot size: The urine spot size range used for analysis. Spots less than or exceeding the user-determined bound limits is ignored during analysis. The value must be in the format of "<*lower bound*> - <*upper bound*>". Units are centimeters unless otherwise specified by the "Area Units" parameter at the bottom of the window. This custom setting can eliminate false positives spots (for example, very small spots that correspond to experimental artifacts that are not considered representative of true voids).

Circularity: The circularity range for analysis. A value of 0 is a straight line, while 1 is a perfect circle. Spots less than or exceeding the range are ignored. The value must be in the format of "<*lower bound*> - <*upper bound*>".

Bins: Spots are binned based on area. If a spot has an area of 0.4px^2, it is placed in the 0.2-0.5 bin. Units are square centimeters unless otherwise specified by the "Area Units" parameter at the bottom of the window. Thresholds must be separated by a dash with no spaces. Zero is always the first threshold. For example, do not input "0.5-1.0-1.5," instead input "0-0.5-1.0-1.5." The purpose of this setting is to quantify shifts in the overall pattern of voiding behavior. For example, diabetes mellitus might shift the pattern from small voids to large and bladder outlet obstruction from large voids to small. Users can quantify these voiding pattern changes by examining changes in average spot size or, using binning, changes in the frequency distribution of void size.

% Offset Center: The percent area defined as the paper's center. A 30% offset indicates that 30% of the paper's area is considered to be the center. This setting enables the user to calculate which percent of the overall urine volume was spotted in the paper's center. This user input might be useful for assessing potential changes in continence (physiologically normal mice tend to void at the periphery of the papers and not in the center).

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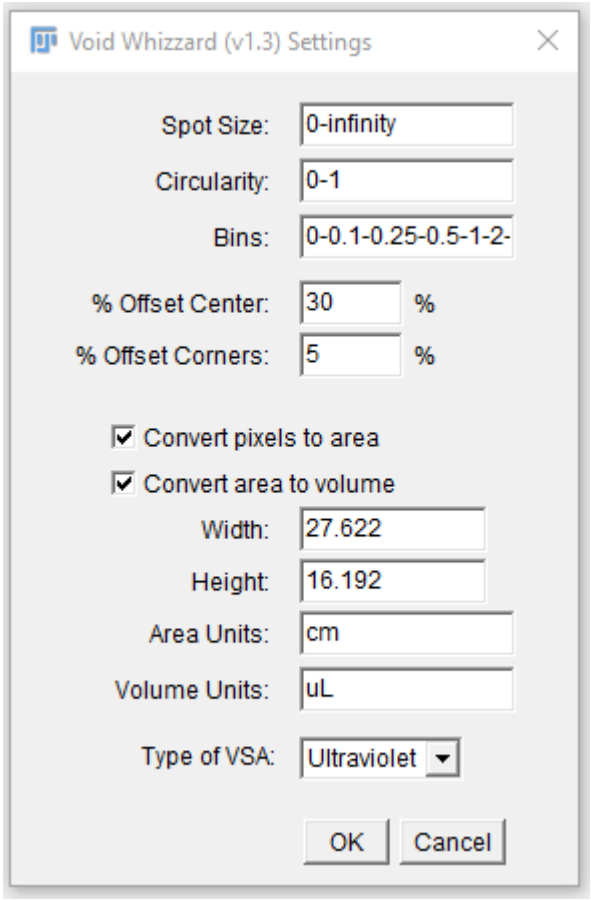
% Offset Corners: The percent area defined as the paper's corners. A 5% offset indicates that 5% of the paper's area is defined as corners. This setting enables the user to calculate which percent of the overall urine volume was spotted in the paper's corners. This user input might be useful in conjunction with center voiding to quantify changes in voiding pattern from periphery to center.



Convert pixels to area: Selection of this checkbox will convert the urine spot areas will be listed as distance measurements (centimeters) instead of pixel measurements. This conversion leverages the paper dimensions entered in the "Width" and "Height" boxes below. If this box is selected, unit label corresponds to the "Area Units" box . If this is not selected, all results will be reported in pixel units.

Convert area to volume: If this checkbox is selected, urine spots will be measured as volumes instead of areas. If this box is selected, the user must include the conv.txt file in the directory containing the raw images. The conv.txt file is not required when 'convert area to volume' is not selected.

Width/Height: Enter paper dimensions. These values are used for the conversion for pixels to area.

Area Units: Enter the paper dimension units here. Spot area output is listed in these units of measurement.

Volume Units: Enter units of measurement associated with the conv.txt file here. Spot volume output is listed in these units of measurement

Type of VSA: Determines how the image will be converted from grayscale into a binary image. If the image contains white urine spots on a black background, select "Ultraviolet". If the image black urine spots on a white background, select "Ninhydrin".

Once you are done entering information, click 'OK'.

7) Select input directory.

When the user settings window closes, another will open prompting for the input directory. This directory contains cropped images from step 4 above and optionally, the conv.txt file. Select the directory to initiate the macro. The Void Whizzard should take approximately eight seconds per VSA Image.

While the macro is executing error messages may appear. This is normal. Ignore these messages and allow the macro to complete execution.

8) Check images.

Once the macro has finished executing, a window will appear with the message “The Void Whizzard has finished executing.” The binary VSA images will be saved in a new folder named “Binary”, which is created within the input image directory by the Void Whizzard.

9) View Data.

Once Void Whizzard finishes executing, the raw data will be saved as “Summary.csv” in the input image directory. The file can be opened in Microsoft Excel or any other spreadsheet software. Each VSA is summarized in a single row. Additionally, the binary image files and ellipse overlays generated by Void Whizzard are saved in a folder named “binary”.

Ellipses fit to spots can be viewed using the FIJI ROI Manager. To view the fitted ellipses for any given image, navigate to the Void Whizzard input folder. Open the desired image in FIJI. Next, press the “T” key to open the ROI Manager. Using the ROI Manager, go to “More>>Open…” and navigate to the “binary” folder created by Void Whizzard. Select the zip file corresponding to the image you opened. This loads all of the ellipses into the ROI Manager. Check the “Select All” box to view all of the ellipses overlayed to the VSA paper image. To export this overlay simply press the flatten button in the ROI Manager and export the resulting image as a .TIFF image.

10) Citation

Void Whizzard is free and open-source. To maintain this status please remember to cite this tool in all publications:

Wegner KA, Abler LL, Oakes SR, Mehta GS, Ritter KE, Hill WG, Zwaans BM, Lamb LE, Wang Z, Bjorling DE, Ricke WA, Macoska J, Marker PC, Southard-Smith EM, Eliceiri KW, Vezina CM. Void spot assay procedural optimization and software for rapid and objective quantification of rodent voiding function, including overlapping urine spots. *Am J Physiol Renal Physiol*. 2018 Oct 1;315 (4):F1067-F1080

FREQUENTLY ASKED QUESTIONS

Is Void Whizzard really free?

Yes. Void Whizzard is open-source software developed by researchers at the University of Wisconsin—Madison.

What are the software requirements for Void Whizzard?

Void Whizzard is a plugin designed for use in FIJI, a redistribution of ImageJ. FIJI is available for free download at https://imagej.net/Fiji/Downloads.

What if mice chew holes in the paper?

Depending on the location of the holes, use the 'fill' function of any image manipulation software to perform a black or white fill in the raw image. Which color is determined by whether the user wants the hole counted as part of a urine spot or as part of the background.

The Void Whizzard binary outputs are not marking all of the urine spots or are only marking portions of spots.

This inaccurate quantification is typically due to noisy or low resolution input images. We suggest increasing the resolution of image capture or adjusting image capture settings to maximize the contrast between urine-stained and unstained paper.