1. Installation
   * Make sure that you have the latest version of Fiji (recently updated or a new install via <http://fiji.sc/#download>). Note that the Fiji ‘installation’ is just a directory that can be copied anywhere (just avoid the standard ‘Program Files’ directory to prevent issues).
   * Copy the files of plugin to the ‘plugins’ directory of your Fiji installation.
   * You will need an extra file which can be downloaded from <http://opencv.org/releases.html> . Choose the ‘Win pack’ of the 3.2.0 release.
   * Execute the downloaded file and point it to any installation directory you’d prefer. Once again, avoid ‘Program Files’ to stop Windows from causing trouble.
   * Go to Control Panel\System\About (Windows 10) or Control Panel \System and Security\System (Windows Server 2008)
   * (Windows 10 only) Click on 'System info' (right side, bottom) and on the popup click on 'Advanced system settings' (left side).
   * Click on Change Settings.
   * Go to “Advance” tab.
   * Click on Environment Variables.​
   * At System variables (lower half) find and click the variable named ‘Path’ and click the 'Edit...' button.
   * In the next popup click the 'New' button first and the 'Browse' button next.
   * Browse to the directory in which you installed OpenCV and select the directory 'opencv\build\java\x64' and click 'Ok'
   * Click 'Ok' again to finish editing variables and 'Ok' to close the System Properties.
   * Next to install the Pathomation support program, go to <http://free.pathomation.com/download/> and download PMA.start. You will probably need to log in via your Google, LinkedIn or Facebook account to do so.
   * Run the downloaded PMA.start.setup . Just click ‘Next’ and ‘I agree’ when prompted. For the install directory, any directory (including the default one) is fine.
   * Start PMA.start (there should be a shortcut on your desktop).
   * This will either open a browser window or add a new tab to an already open browser. Please make sure that you log in if the opened PMA web page asks you (not always the case). If the page is done with initializing, you can ignore it from now on.
   * Note that PMA.start will also start a small window in which you can restart the web page. You can ignore or even minimize it.
2. Settings

* Open the settings by going to meu item Plugins > A Steatosis Detector > Steatosis Detector Settings.
* This will open an example image and a settings dialog.
* In the dialog you can change the minimum and maximum settings that will determine if a white area in the image has the correct properties to qualify as steatosis. Also you can determine the threshold in which a pixel in the image is still considered white.
* The minimum area will remove any white area that is smaller than the given number of square micrometers from consideration. Please note that in the manual mode, these areas cannot be retrieved.

This setting is meant to reduce the often very large number of very small areas that may occur for any reason other than steatosis. To get an accurate number, examine a typical piece of steatotic tissue and measure the smallest real steatotic area you find.

We found 29 to be a good lower limit.

* The maximum area will automatically dismiss any white areas larger than the given value. Note that in the manual mode the label of these areas can be changed to steatosis if need be.

The maximum value is used to get rid of tearing and the white background outside of the tissue areas. Our max value is typically set to 6000

* The saturation threshold determines which pixels are considered white. This threshold is reflected in the example image and changing it via the slider (not the number field!) will update the image accordingly. Please have patience as this may take some time.

The main point of this setting is to determine where the steatosis ends and where the tissue starts. Look at the example image to make a good judgement.

For our own images we used a value of 25.

* The final setting that can be used is the checkbox to enable the two step core saturation threshold. When judging the right saturation value in the previous step, you might have chosen a high value to include as much of the steatosis edge as possible. The drawback of this is that this will also increases the chances of finding areas that only contain the higher allowed saturation values and no clear white background (i.e., these areas are slightly pink all over). To avoid these areas, you can switch on the two step core saturation which enforces that any steatosis area has at least a small (12 area of very low saturation (< 15). Note that this is not reflected in the example image.
* Press OK to save the settings.

1. Manual steatosis measurements
   * For every slide you want to measure, you will have to start the plugin. You can start it by selecting the Plugins menu in Fiji and from there select the ‘A Steatosis Detector’ menu and the Steatosis Detector plugin within.
   * In the following popup, navigate to the slide image you wish to measure and select it. Click ‘Open’ to proceed.
   * Occasionally, the first start of the plugin after starting Fiji will fail with an error. Please ignore it (click away the error window) and try again.
   * The next popup will show the slide image zoomed out. By clicking the left mouse button on the image and dragging the mouse while holding the left button depressed, you can select a rectangular area of interest. Clicking outside a selected area will remove it and you can reselect if you want to.
   * Please select and area that contains all of the tissue you wish to measure. This does not need to be very precise, just make sure that all of the tissue is within the selection. Please do note that smaller selection will compute faster than a large one, so try and stay somewhat close to the tissue.
   * Click ‘Ok’
   * If you have used the manual selection before on this image, the tool has saved your selection in the ‘Steatosis images’ folder in the same folder as the image. It will assume that you need the same selection again and will skip the selection process described above and immediately proceed with the annotation as per below. If you wish another selection to be made, please remove the ROI and tif file for this image from the ‘Steatosis images’ folder.
   * Next you will see the selection cut-out as an image. Wait while the program is calculating. You will see the cut-out image change into just a black and white image. This image is your work in progress image. A red square will tell you what part of the image you are currently working on. The square will move from left to right, top to bottom over the image while you work. It will skip over completely black parts (no tissue in there).
   * The program will now quickly open and close some images while processing the part of the image as given by the red square. Please wait until you see a pop-up ‘Roi removal’.
   * If you are using the manual tool just to create an ROI for the automated run (see below), please choose Cancel at this point. The ROI has been saved already.
   * The image shown is the part of the image as designated by the red rectangle and all potential steatosis areas have been coloured bright green. Any areas coloured light blue are white tissue areas that have been qualified as non-steatotic by the automated steatosis finder algorithm.
   * Please check the selected areas:
     + If a green area is actually just background, click on it with the left mouse button. This will remove the green selection and the area will be ignored by the program.
     + If a selected area is tissue, but not steatosis, hold the Alt button and left click the area. Its colour will now change to light blue. This signals that the program will still count it as part of the tissue area, but not as an area of steatosis.
     + If you made a mistake and set an area to non-steatosis tissue (light blue) while it was steatosis, hold the Ctrl button and left click the area. This should change the colour back to green. There might be a small blue halo left in the area. This is just a visual glitch and you can ignore it.
     + If you completely deselected an area as background, you can retrieve it by holding the Shift button and left clicking the area. This will restore the selection colour.
     + If you first selected an area as non-steatosis tissue and then removed it and both actions were wrong, please first restore the selection and then set it back to steatosis as described above.
   * Note that larger white areas that have not been coloured green or blue (and are therefore non-tissue background) might have been automatically dismissed by mistake. In such a case, these can be retrieved as a tissue area (steatosis or not) by shift-clicking them.

This is not possible for very small white areas. Please update your settings (see above) if too many of these are dismissed that should have been steatosis. Also note that these areas are considered part of the tissue and not simply background.

* The choice dialog offers several useful options in the form of checkboxes:
  + The show selections checkbox can be used to switch off the colouring of the selected white areas. This can help in judging the type of area (i.e. steatosis, other tissue or bbackground).
  + The ‘Filled ROI’ box can be used to show the selected areas only by their coloured outline.
  + By checking the ‘All fot’ or ‘All non-fat tissue’ boxes you can change all the selections to either steatosis or non-steatotic tissue respectively. Please note that this cannot be undone by merely unselecting the boxes. Manual correction are still possible after the use of either ‘All’ box.
  + If you are done with judging the selections, press the ‘Done’ button on the pop-up.
  + The program will now calculate the next square. Please wait until you get the ‘Roi removal’ pop-up again.
  + This will continue until the red square on the work-in-progress image has reached the right bottom of the image. When that happens all images will close. You can prematurely stop the process by selecting ‘Stop and Save’ or Cancel. The former adds the current set of selections to the results file, while Cancel will not. Note that any previous parts of the image that have been handled by choosing Done will already have been saved.
  + You can find the results of your measurements in the ‘Steatosis results’ directory which is a sub-directory of the spot where your original image is located. Within that folder will be a .xls file of the same name as the image.
  + In the .xls file you can find the total tissue area, total fat area and the percentage of tissue that is fat. Also, the total number of fat areas, non-fat white tissue areas, and background white areas is given as well as the actual size for each of these areas.
  + In the ‘Steatosis images’ folder you can find the ROI you used on the whole slide image to determine which part needed to be processed as well as a tif image file of the selected area.

1. Automated steatosis measurements
   * Create a folder which contains all the images that you wish to measure.
   * For all images, run the manual selection until the step that tells you to cancel for the automated process. This is needed to establish the region of the whole slide image that the automated tool will use. Note that you only need to run this step once. If the ROIs have been selected, numerous automated runs (with different parameters for example) can be done on the same image selections.

If you need to redo any selection, please remove the tif and ROI file of that image from the ‘Steatosis images’ folder.

* + Select the ‘Steatosis Detector Automated’ plugin from the ‘A Steatosis Detector’ menu in Plugins.
  + The plugin will ask you to select a folder to process. Please select the image folder you prepared and press Ok.
  + The tool will now start to process all the images in the folder without any further input needed. This may take quite a while. Please be careful in using your computer in the meantime as Fiji will sometimes get the focus of the operating system if a new image pops up. If you are typing at that time, Fiji may interpret this as commands and this could potentially derail the automated process.

1. Some remarks:
   * Please note that the program pre-filters the possible fat areas on size. To small areas will be considered background noise in the image and will be ignored. During the running of the program you may see the total ‘fat’ areas found for each image square. These are the pre-filtered and pre-reviewed areas, so the result in the .xls file will probably be (a lot) less areas.
   * Fiji is a touchy program. When the program is calculating, please do not click on any image (to move it out of the way for example). This will cause Fiji to focus on that image, which may result in errors.
   * When the ‘Roi removal’ popup is showing, the program is waiting for user input. At this time it is possible to move around images/popups/tables without disrupting the program.
   * Depending on you version of Windows and your settings, any Fiji windows that are hidden behind a wall of other windows can be found by hovering over the Fiji icon on the Windows task bar. This will show you a selection of windows and you can chose one to bring to the front.
   * Any remark on how to improve the program and user-interaction especially is appreciated. Let me know.