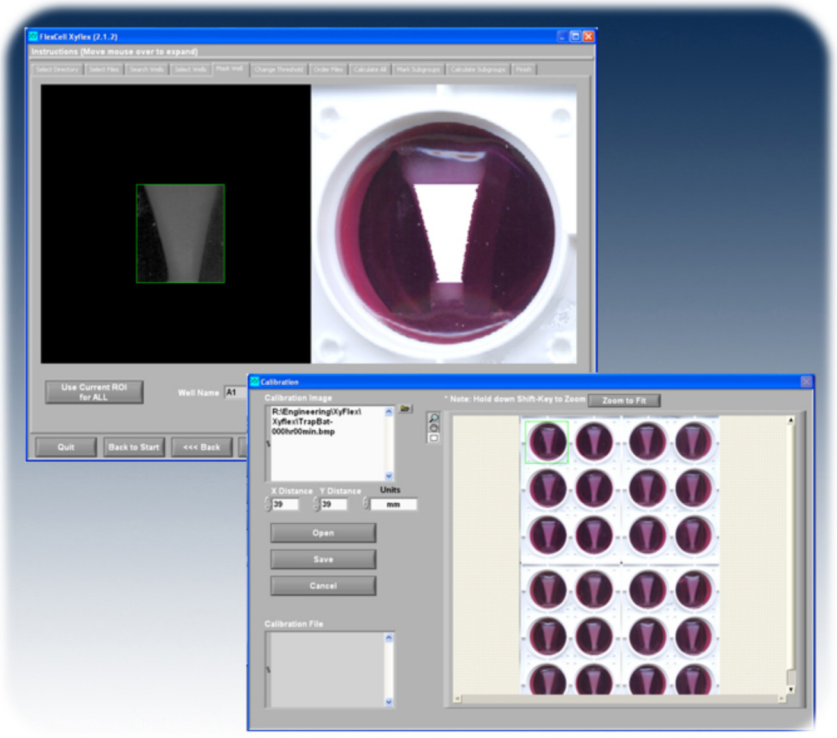
USER MANUAL

XyFlex™

AREA MEASUREMENT SOFTWARE



05-12-17
XYFLEX 3.3.0
Rev 5.0

Culturing Cells in a Mechanically Active Environment™

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1. INTRODUCTION

1.1 USE OF XYFLEX™ SOFTWARE WITH TISSUE TRAIN® CULTURE PLATES

XyFlex™ software is designed to automate the area measurement of a series of images. It was initially designed to evaluate the area compaction of 3D bioartificial tissue constructs fabricated using the Flexcell® Tissue Train® Culture System. The ScanFlex™ scanner and software enable the user to create scanning regimens so bioartificial tissues in 6-well or 24-well Tissue Train® culture plates can be scanned periodically to determine the rate of matrix compaction by cells over time. The XyFlex™ software examines area measurement of the bioartificial tissues in the image series. The software creates Excel and Text files, which may be used to evaluate the change in area measurement of the analyzed data.

1.2 USE OF XYFLEX™ SOFTWARE FOR OTHER APPLICATIONS

XyFlex™ software may also be used for other applications requiring area measurement. The images must be captured by the Flexcell® ScanFlex™ software and scanner. The component also should be analyzed inside of the 6-well or 24-well plate templates provided by Flexcell due to the region of interest (ROI) locations. The 6-well orientation will examine up to 24 positions in a series of images. The 24-well orientation will examine up to 96 positions in a series of images.

2. COMPONENTS

- XyFlex™ software V3.3.0
- User manual
- Desktop computer or laptop computer (not provided). See Table 1 for more details.

NOTE: *Table 1 has minimum system specifications for the computer to operate the scanner. More memory will be needed depending on scanner frequency and image resolution (see Table 2 in the Appendix for more information on image size corresponding to image parameter settings).*

Table 1. *Minimum system requirements for XyFlex™*

Minimum system requirements: USB 2.0/1.1	**CPU**	**RAM**	**HDD**
Microsoft Windows XP, Vista, and Windows 7 (32-bit)	1 GHz	1 GB	95 MB
Microsoft Windows XP, Vista, and Windows 7 (64-bit)	1 GHz	1 GB	95 MB
Minimum screen resolution	1024 × 768 pixels		

3. INSTALLING XYFLEX™ SOFTWARE

1. Insert the XyFlex™ CD into the CD-ROM drive of the computer.
2. The XyFlex™ installation process should start automatically. If not, go to "My Computer" and double-click the CD-ROM drive.
3. Double-click the "Setup" icon (Fig. 1).

4. Select the destination directory to save all program files (Fig. 2) and click *NEXT* to continue.
5. Accept the National Instruments license agreement and click *NEXT* (Fig. 3).
6. Click *NEXT* to begin the installation (Fig. 4).
7. Click *NEXT* after the installation is complete to continue (Fig. 5).
8. Activate the National Instruments product through a secure internet connection (Fig. 6) and click *NEXT*.
9. The NI Activation Wizard will prompt to enter the serial number for the "Vision Development Module 2011 Vision Run-Time" (Fig. 7). Input the Serial Number located on the NI Vision Development Module Run-Time License, which is included with the equipment documentation.
10. After entering the Serial Number and activating the software, select *FINISH* (Fig. 8).

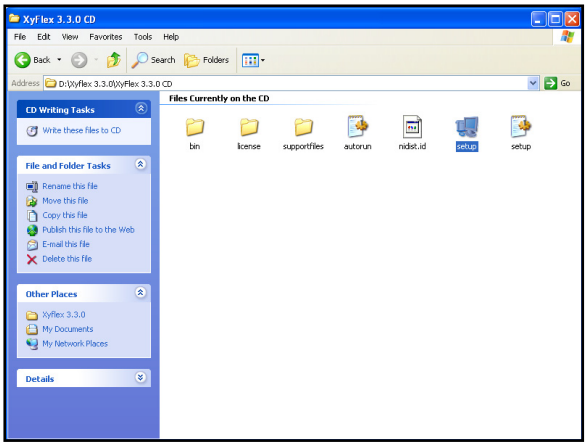

Figure 1. *XyFlex™ CD contents.*

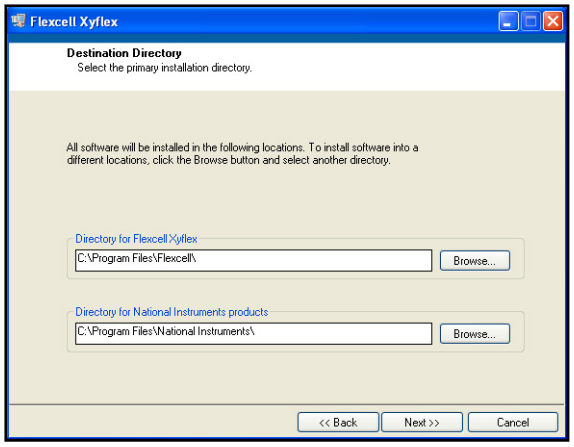

Figure 2. *Destination directory.*

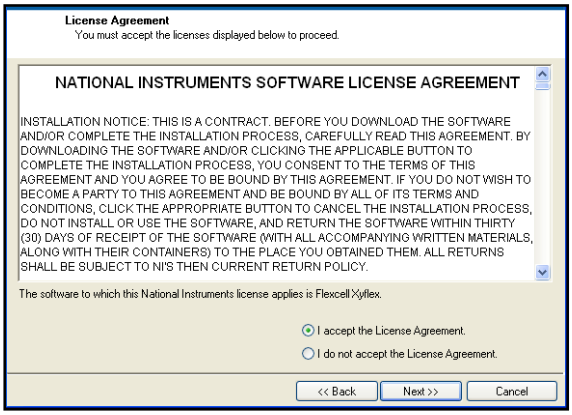

Figure 3. *NI license agreement.*

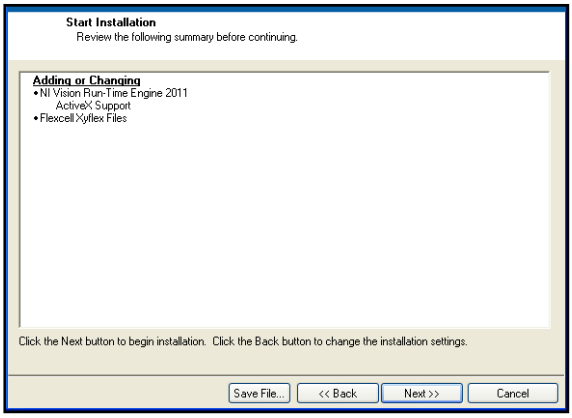

Figure 4. *Starting XyFlex™ installation.*

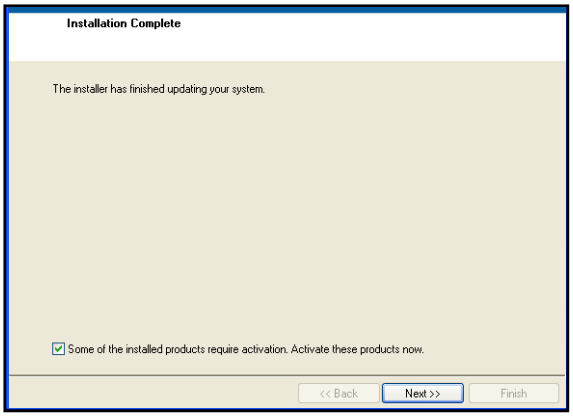

Figure 5. *Installation complete.*

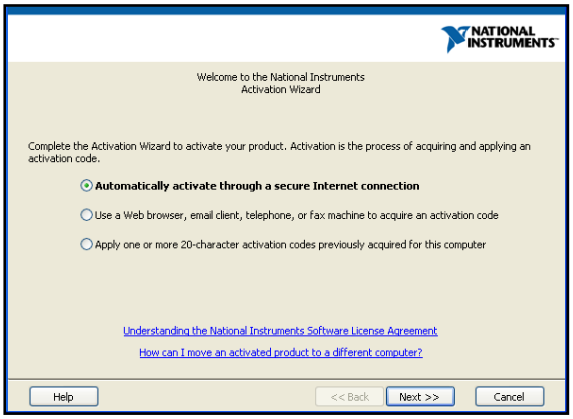

Figure 6. *NI activation wizard.*

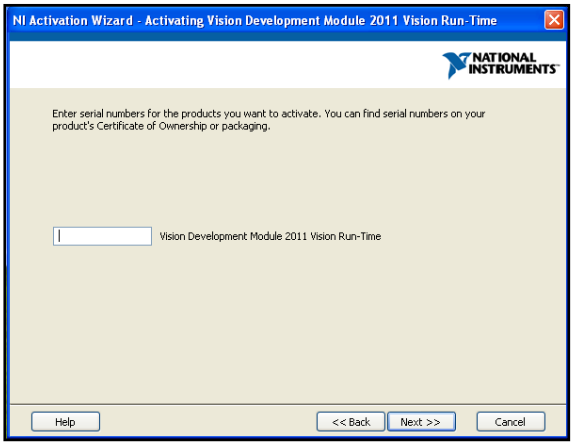

Figure 7. *Vision activation code.*

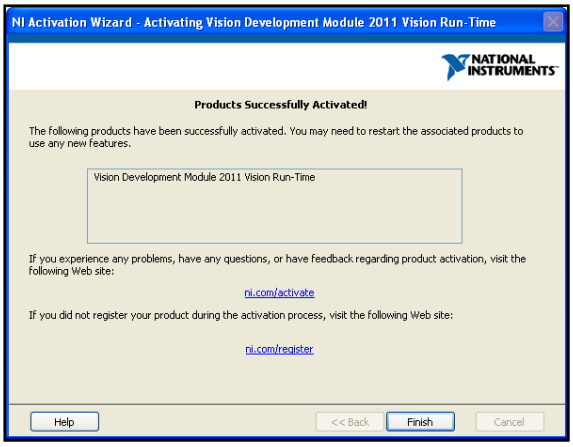

Figure 8. *Successful activation screen.*

3.1 ACTIVATION OF XYFLEX™ SOFTWARE

1. Click on the XyFlex™ icon on the desktop or go to program files and open XyFlex™ software.
2. When XyFlex™ activation screen opens up, select either *ACTIVATE XYFLEX* or *EVALUATE XYFLEX* (Fig. 9).

Figure 9. *Options for activating XyFlex™ software.*

3. If *ACTIVATION* is selected, two user codes will be displayed in the next screen (Fig. 10). Call (1-800-728-3714 or 919-732-1591) or email (*activation@flexcellint.com*) at Flexcell® International with the user codes to obtain an activation code.

Figure 10. *User code and activation code for XyFlex™ activation.*

4. Note: Do NOT close the XyFlex™ software until the activation codes have been received from Flexcell® International. Doing so will generate new user codes, voiding the previous user codes and the activation code.
5. Enter the activation code in *ACTIVATION CODE 1* box and click on *CONTINUE*.
6. If *EVALUATE XYFLEX* is selected, you will have 30 days to use the XyFlex™ software after which it will automatically deactivate.

3.2 GENERAL SETTINGS

The XyFlex™ software may be installed on any Windows based PC meeting or exceeding the requirements listed in Table 1. The ScanFlex™ and XyFlex™ software do not necessarily have to be installed on the same computer, but the images to be analyzed must be present on the computer with the XyFlex™ software. The computer with the XyFlex™ software also should have the following settings turned OFF for long term image series evaluations:

- Auto updates
- Virus scanning
- Sleep mode
- Automatic defragmentation.

The maximum number of images to be analyzed is only limited by the memory capacity of the computer.

NOTE: *The image resolution determines the size of each file, so make sure to record the resolution of all images captured before evaluation.*

The approximate file size in MB of the scanned images with respect to the file type, resolution, and quality (*JPEG is the only image type which uses quality) are listed in the Appendix in Table 2. Always be certain that you have sufficient memory on the hard drive to contain all the images.

3.3 ORIENTATION AND ANALYSIS OF THE TISSUE TRAIN® CULTURE PLATES

Two different plate frames, one frame for four 6-well plates and one frame for four 24-well plates, are provided with the ScanFlex™ software package. The culture plate location in the frame corresponds to the numbering of the plates and wells in the XyFlex™ software. If the plates are placed according to the instructions, the wells will be named as shown in Figures 11-14 in the XyFlex™ software. See the Appendix for an illustration to identify the corresponding location of wells on the scanned image with reference to their physical orientation on the scanner bed. The orientation is important regardless of whether the Tissue Train® culture plates are utilized or if the image series analyzed is for a customized experiment.

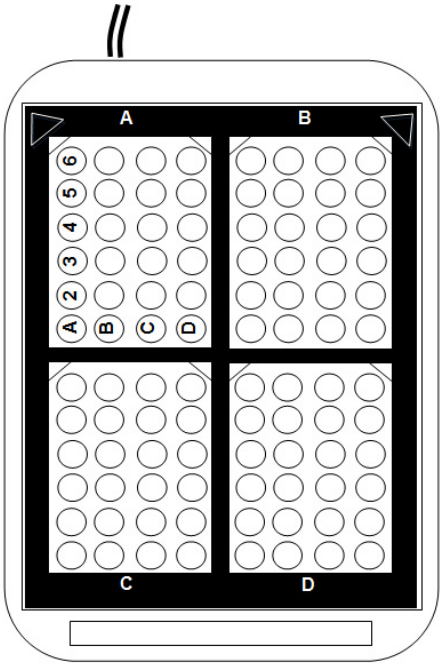

Figure 11. *The orientation and numbering of 24-well culture plates on the scanner when the frame provided is used for placing the culture plates on the scanner. The black area is the frame used to hold the plates on the scanner surface.*

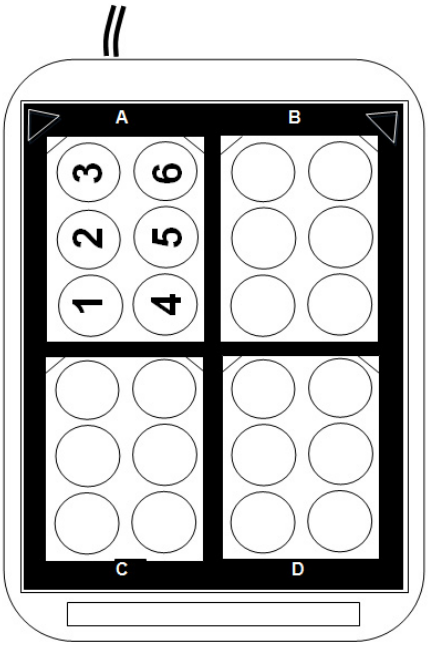

Figure 12. *The orientation and numbering of 6-well culture plates on the scanner when the frame provided is used for placing the culture plates on the scanner. The black area is the frame used to hold the plates on the scanner surface.*

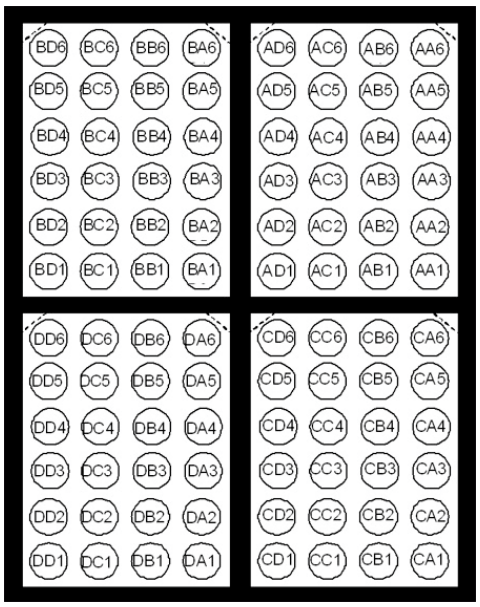

Figure 13. *The orientation and numbering of 24-well culture plate wells in the scanned picture. The black area is the frame used to hold the plates on the scanner surface. This numbering system is used in the XYFLEX™ software for identification of wells.*

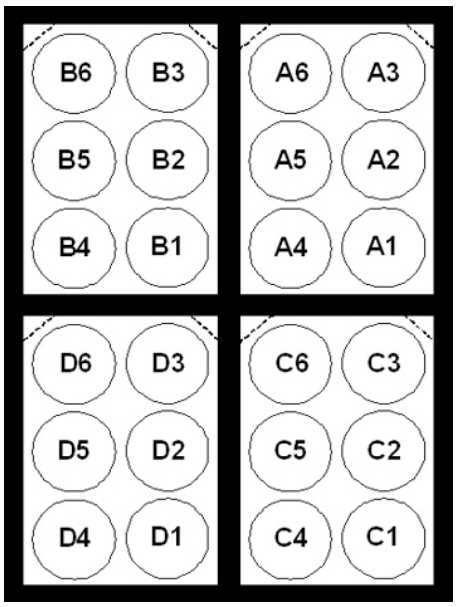

Figure 14. *The orientation and numbering of 6-well culture plate wells in the scanned picture. The black area is the frame used to hold the plates on the scanner surface. This numbering system is used in the XYFLEX™ software for identification of wells.*

3.4 OPENING THE XYFLEX™ SOFTWARE

When the XyFlex™ software is completely installed, the XyFlex™ icon (Fig. 15) is displayed on the desktop. Click on the icon to open the XyFlex™ software. The XyFlex™ *SELECT FOLDER* screen (Fig. 16) will open.

Figure 15. *XyFlex™ Icon.*

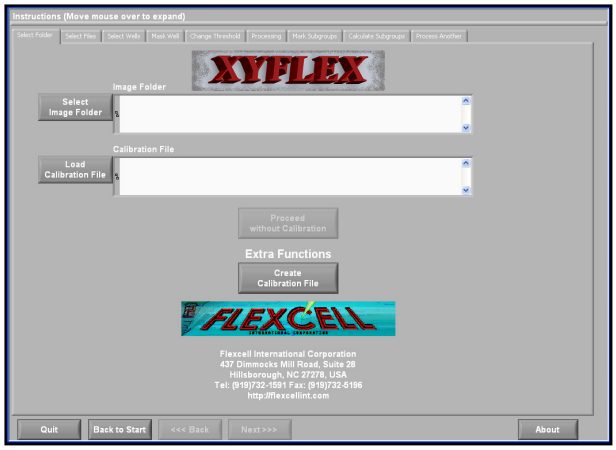

Figure 16. *XyFlex™ start-up screen.*

4. XYFLEX™ PRE-PROCESSING

The XyFlex™ software will begin the area measurement analysis in the pre-processing stage. This stage begins on the start-up screen (Fig. 16).

1. The *IMAGE FOLDER* window is located below the XyFlex™ logo. Click *SELECT IMAGE FOLDER* to open the directory where the images to be analyzed are located. Once in the directory, select *CURRENT FOLDER* located in the bottom right corner of the window.
2. The selected directory should appear in the XyFlex™ window (Fig. 17) next to the *SELECT IMAGE FOLDER* button.
3. Next, the user may choose whether or not to load a *calibration file*. The *calibration file* is used to define a known distance in respect to the specific number of pixels in a specified area of an image to create a ratio for processing.
   a. Selecting *LOAD CALIBRATION FILE* will allow the user to load a pre-defined calibration (*.xml*) file created during a previous analysis. Choose the desired (*.xml*) file. The file will load into the *CALIBRATION FILE* window. Next, go to Section 5.2 of this manual.
   b. Selecting *PROCEED WITHOUT CALIBRATION* will allow the user to continue the analysis using only pixels as the unit of area measurement. To *PROCEED WITHOUT CALIBRATION*, go to Section 5.2 of this manual.
   c. To use area measurement units of μm^2, mm^2, or cm^2, choose *CREATE CALIBRATION FILE,* which will take the user to the Calibration screen (Fig. 18).

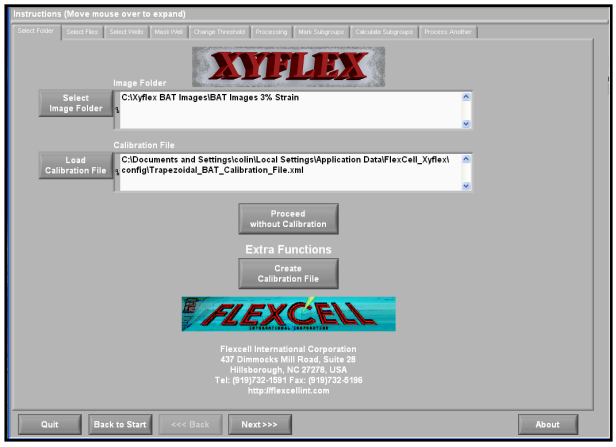

Figure 17. *Image folder and calibration file selection.*

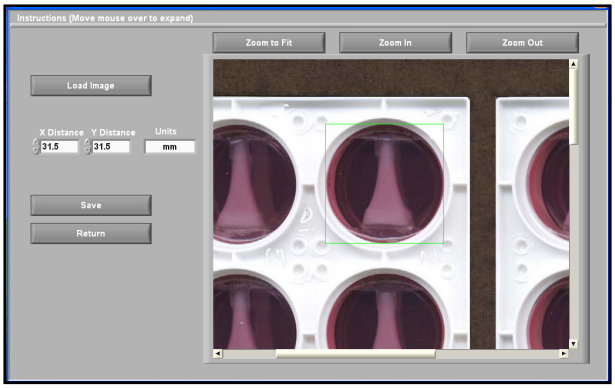

Figure 18. *Screen to create a calibration file.*

4.1 CREATING A CALIBRATION FILE

Calibration file accuracy is vital to obtaining correct post-processing results.

1. Select *LOAD IMAGE* to open an image where a physically measured dimension is known. For example, a user may choose to place the highlighted green rectangle to contain the boundary of one 6-well plate in the scanned image. If the user measures the 6-well plate using a set of calipers and the resulting dimensions are 92.2 mm x 136 mm, the *X DISTANCE* window will read 92.2, the *Y DISTANCE* window will read 136, and the *UNITS* window will display *mm*.
2. Select *SAVE* and choose the directory to save the calibration file *(.xml)* for processing the images. The default save directory is **C:\Documents and Settings\All Users\Application Data\Flexcell_XYFLEX\config\"Calibration_File_Name_Chosen_by_User".xml**.
3. Select *RETURN* to go back to the *SELECT FOLDER* screen. The calibration file directory will appear in the *CALIBRATION FILE* window. Select *NEXT* to proceed to the *SELECT FILES* screen.

4.2 DEFINING THE IMAGE FILE PROCESSING PARAMETERS

The *SELECT FILES* screen allows the user to arrange the files in the order which they will be processed, choose the *WELL ORIENTATION*, select the *RESOLUTION* of the images captured in the SCANFLEX™ software, and choose an *ANCHOR FILE*.

4.2.1 Ordering the Image Files

1. The images from the *IMAGE FOLDER* appear in the left window on the *SELECT FILES* screen (Fig. 19). The images to process may be selected one at a time or grouped using the Control or Shift keys. They are moved to the right window using the (>) button. Use the (>>) to move all the images to the right window. The right window signifies the images that are going to be processed. *The images are processed in the same order as they appear in the window.* To deselect an image from being processed, select an image in the right window or group them using the Control or Shift keys and press the (<) button. The (<<) moves all the images back to the left window to remove them from processing.

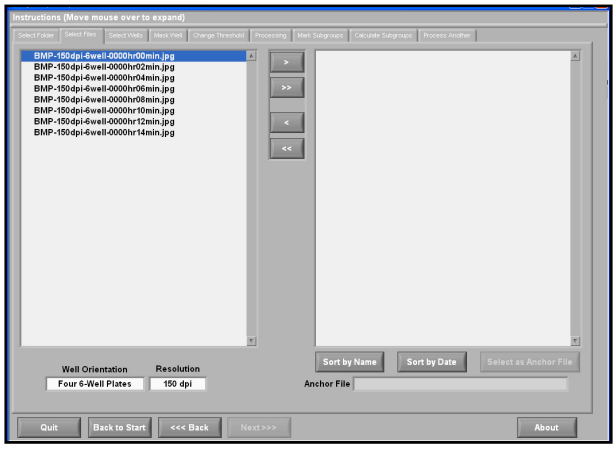

Figure 19. *Selecting the image files to analyze.*

2. Once the files are in the right window, they may be ordered using *SORT BY NAME* or *SORT BY DATE*.

***NOTE**: Make sure to pay close attention to the file names and the times in the image descriptions so the images will be in the desired order.*

3. The user may also manually move image files by using the mouse to select the file in the right window and drag the file up or down.

4.2.2 Choosing the Well Orientation

The *WELL ORIENTATION* is the type of plate the images reflect and allows the software to find each well automatically for analysis. The choices are *FOUR 6-WELL PLATES* or *FOUR 24-WELL PLATES*.

4.2.3 Selecting the Resolution

The ScanFlex™ software allows the user to capture the image in *150 dpi, 300 dpi, or 600 dpi*. The user should record the image resolution chosen when scanning occurs. The software automatically locates the center of each well in respect to the *RESOLUTION* selected.

4.2.4 Selecting the Anchor File

The *ANCHOR FILE* is generally the first image in the series. This file will be the image where threshold values and the region of interest (ROI) are initially defined for processing the rest of the images in the series. The threshold values are the upper and lower limits of color contrast. The ROI is the area in which the threshold values are applied. These processes will be covered in *Sections 5.4 and 5.5,* respectively. Double-click the desired anchor file name in the right window, and the file name will appear in the *ANCHOR FILE* window (Fig. 20).

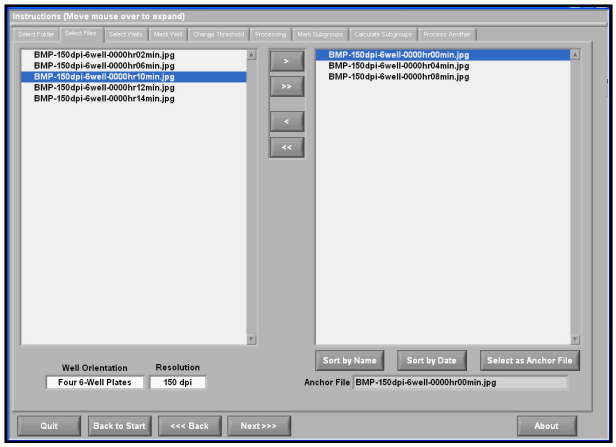

***Figure 20.** Selecting the image files to analyze.*

4.3 SELECTING THE WELLS TO ANALYZE

The well positions of the scanned images are in the orientation shown in Figure 21 for the 6-well and 24-well plates.

The *SELECT WELLS* screen (Fig. 22) displays the default labeling of the *WELLS DISPLAYED* for the 6-well plate. The user may alter the *WELL NAME* by typing a new name in the window and selecting *RENAME WELL*. The highlighted well name in the *WELLS DISPLAYED* column will change as a result.

1. To streamline multiple groups of processed images, select *SAVE WELL NAMES* to save the *WELLS DISPLAYED* column to a Text file for later recall.
2. The *LOAD WELL NAMES* button allows the user to upload a group of previously renamed wells in the *WELLS DISPLAYED* column.
3. Selecting the *CLEAR WELL NAMES* allows the user to clear the renamed wells in both the *WELLS DISPLAYED* column and the *WELLS TO USE* column. The names will revert back to the software default well names. After the *WELLS TO USE* column meets the desired criteria, select *NEXT*.

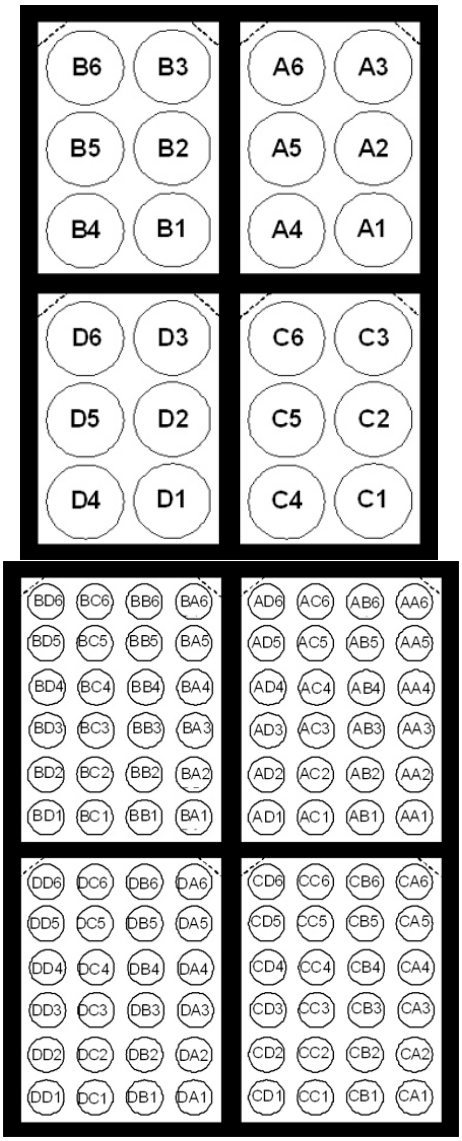

Figure 21. *Well positions in the scanned images for the 6-well and 24-well plates.*

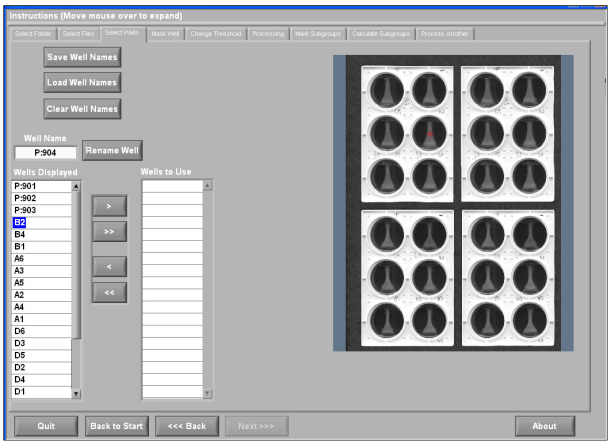

Figure 22. *Selecting the individual wells to analyze.*

4.4 SELECTING THE REGION OF INTEREST

The region of interest (ROI) is the selected area where the threshold is evaluated. The threshold is defined as the pixel contrast coloration located between the upper and lower limits of the desired threshold.

1. The ROI rectangle may be relocated and resized to locate the best fit for the ROI (Fig. 23). For BATs, the ROI is generally located from the end of one Tissue Train® nylon anchor to beginning of the opposite nylon anchor in respect to height and a width slightly wider than the widest measurement of cross section of the BAT.

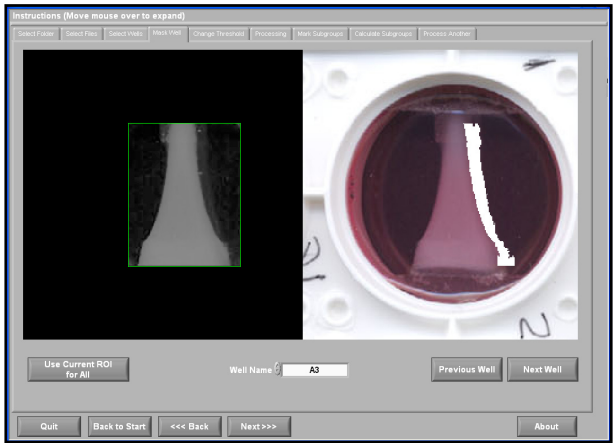

Figure 23. *Selecting the Region of Interest (ROI).*

2. The user may advance the process of selecting the ROI for each of the individual wells (24 wells for a 6-well plate experiment and 96 wells for a 24-well plate experiment) by selecting *USE CURRENT ROI FOR ALL*.
3. The user may choose to select the ROI for each individual well by scrolling to the *WELL NAME*, adjusting the ROI rectangle to the desired area, and then selecting *NEXT WELL* until the process is complete. ***NOTE:*** *the area defined in pixels is shown to the right of the selected*

WELL NAME. Choose *NEXT* to begin the threshold range evaluation.

4.5 CHOOSING THE THRESHOLD RANGE

The threshold range is a determining factor in the effectiveness of the software to complete a successful image series area measurement evaluation (Fig. 24).

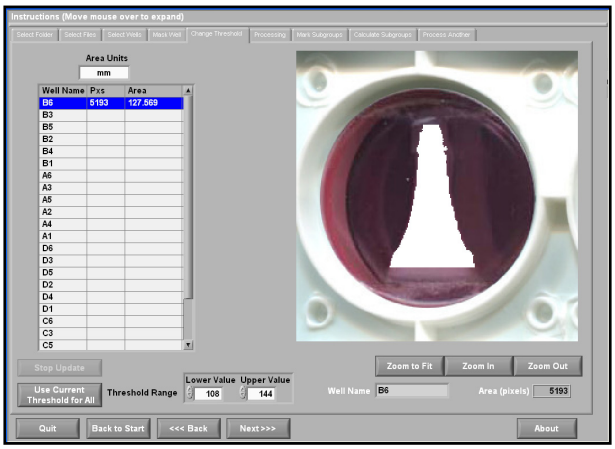

Figure 24. *Adjusting the threshold in the ROI.*

1. The *AREA UNITS* should be chosen first. The units available for selection in the drop down window are *pixels, μm^2, mm^2*, and *cm^2*. The units chosen will be used for the remainder of the analysis.
2. The large window below the area units lists the *WELL NAME, PXS* (pixels), and the *AREA*. The *WELL NAMES* and the order should resemble the well names and order defined during the *SELECT WELLS* screen. ***NOTE:*** *The first row is already filled in at the initial state as a consequence of the LOWER VALUE and UPPER VALUE of the threshold range shown at the bottom of the screen.*
3. Next, the user should adjust the *LOWER* and *UPPER VALUES* of the threshold so the desired area to be measured is completely pixilated with white pixels in the image shown on the right side of the *CHANGE THRESHOLD* screen (Fig. 25). Choosing a *LOWER VALUE* of zero will fill the ROI with 100% white pixilation, meaning 100% of the area inside the ROI is selected in the area measurement evaluation. Choosing an *UPPER VALUE* of 140 will fill the ROI with 0% white pixilation, meaning 0% of the area inside the ROI is selected in the area measurement evaluation. The *LOWER* and *UPPER VALUES* should be adjusted accordingly to obtain the desired result. The user may utilize the *ZOOM IN*, *ZOOM OUT*, and *ZOOM TO FIT* buttons below the window to gain an improved view of the pixilation inside the ROI.

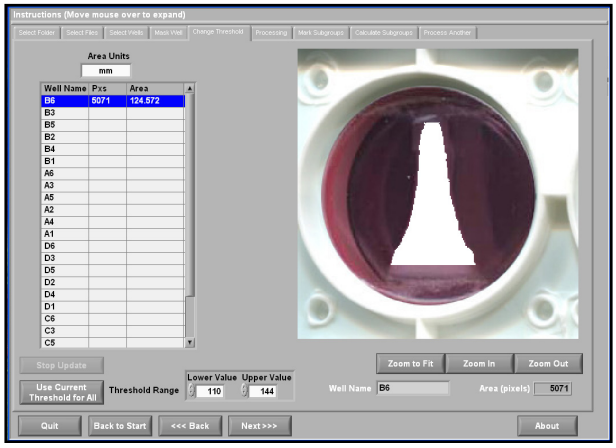

Figure 25. *Evaluating the anchor image threshold.*

4. The user may choose *USE CURRENT THRESHOLD FOR ALL* to employ the *LOWER VALUE* and *UPPER VALUE* selected for the current image for all 24 wells if 6-well plates are used and all 96 wells if 24-well plates are used (Fig. 26). This selection will cause the software to evaluate each well in the anchor image with the current threshold. The user may choose not to use the current threshold for all the wells by selecting *STOP UPDATE* during area evaluation of the anchor image. When the threshold is acceptable, select *NEXT* to continue.

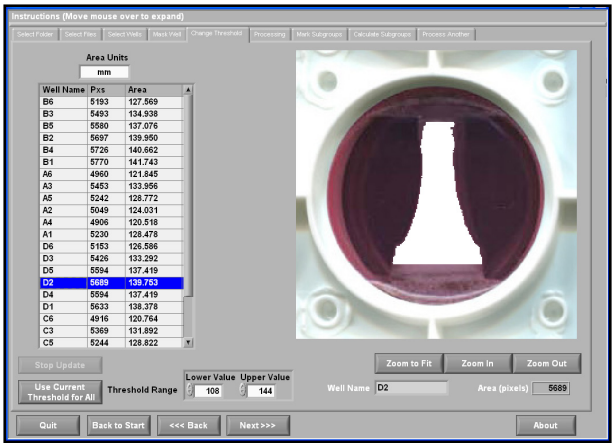

Figure 26. *Processed well images of the anchor file only.*

5. XYFLEX™ PROCESSING

When pre-processing is complete, it is time to process the image series.

1. The user may double-click on the well name in the left column of the window to view the image of the chosen well with the previously selected threshold pixilated. Select *START PROCESSING* to begin processing the image series (Fig. 27). At any point during the processing, the user may stop the analysis by selecting *STOP PROCESSING*. The upper cell in the *00:00* column will highlight red when processing is complete. After processing is complete (Fig. 27), the user has several options for post-processing (see Section 7).
2. The user may scroll up and down using the keyboard to highlight the series of images for a specific well. Selecting *STEP FORWARD* and *STEP BACKWARD* moves the cell selection left or right across the highlighted row. The red highlighted cell represents the image shown in the right window of the *PROCESSING* screen.

NOTE: *The speed of the CPU's processor coupled with the resolution of the scanned image determines the speed the user may scroll through the images. Using the mouse to select cells is more time efficient then scrolling with the keyboard arrows.*

3. When the processed data represents the desired results, the user may choose to *EXPORT TO EXCEL (.csv)*, *EXPORT TO TEXT (.txt)*, or click *NEXT* to move the data into subgroups. The *EXPORT TO EXCEL* action creates a *Comma Separated Values file (.csv)*. Once this *(.csv)* file is saved to a designation, the file may be resaved or reopened and saved as a Microsoft Excel file or opened in other data analysis software programs.

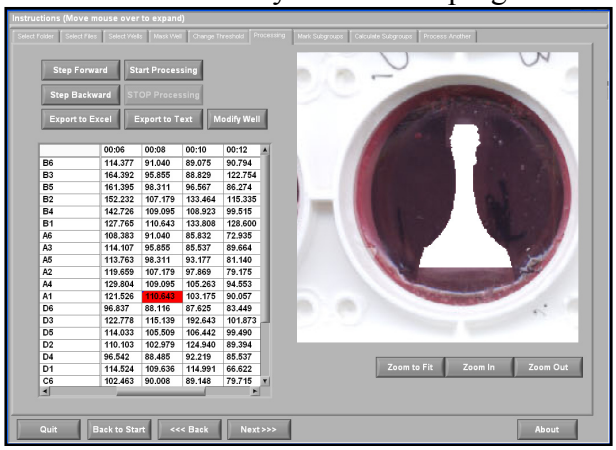

Figure 27. *Processing the image series.*

6. XYFLEX™ POST-PROCESSING

6.1 MODIFYING INDIVIDUAL WELL IMAGES

Individual images in the processed results may require more consideration if the pixilation does not possess the desired effect. For instance, there may be pixels pixilated white that are outside of the object being analyzed, but inside of the ROI. In this case, highlight the cell respective to the well image and select *MODIFY WELL* (Fig. 27).

1. A combination of adjusting the threshold range for a selected well image and utilizing the editing tools allows the freedom of pixilation creation or deletion (Fig. 28). The threshold range for the selected well image may be adjusted using the *LOWER VALUE* and *UPPER VALUE*. The user may also *ZOOM IN* to sections of the image to ensure a higher quality pixel selection in a particular section of the chosen image. The *AREA (PIXELS)* and *AREA (UNITS)* are shown below the image window. The *AREA (UNITS)* are the units chosen by the user during the *CHANGE THRESHOLD* screen, either *pixels, μm^2, mm^2*, or *cm^2*.
2. When the threshold in the image is satisfactory to the user, select *ACCEPT* (Fig. 29). This action will navigate the user back to the *PROCESSING* screen with the updated information from the *MODIFY WELL* screen in the selected cell. Repeat this process until all the well images are acceptable and then select *NEXT* on the processing screen.

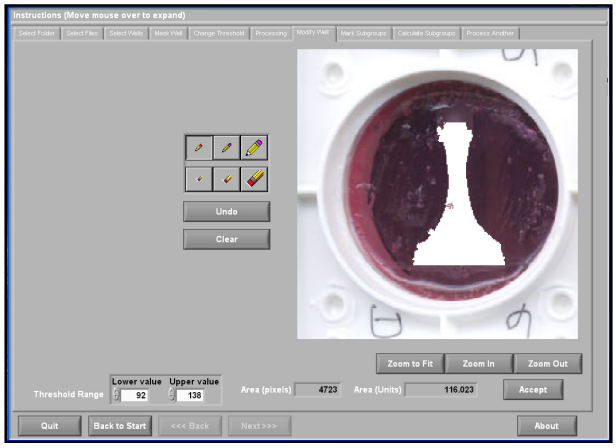

Figure 28. *The processed well image prior to manual editing.*

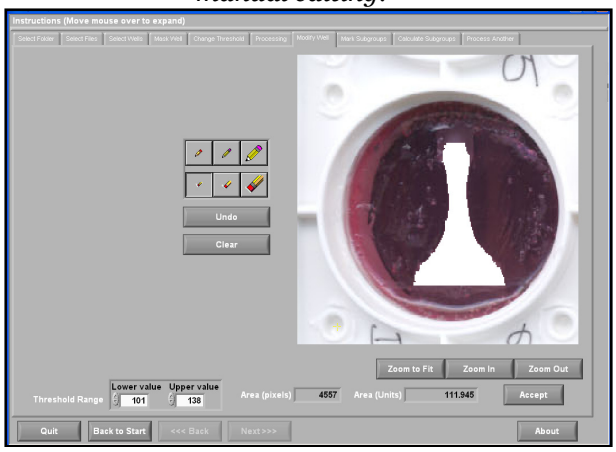

Figure 29. *Adjust the threshold and use the editing tools to modify a specific well image.*

6.2 CATEGORIZE THE WELLS INTO SUBGROUPS

The *MARK SUBGROUPS* screen allows the user to categorize the wells into particular subgroups with group names, if desired (Fig. 30). *This step is completely optional*. Breaking the wells down into subgroups may simplify data extrapolation when analyzing the processed data.

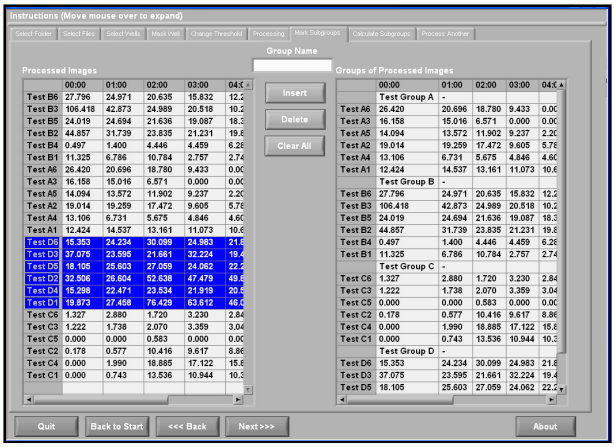

Figure 30. *Create subgroups with the wells.*

1. Type the subgroup name in the *GROUP NAME* window at the top center of the screen.
2. Highlight the wells in the left window to be included in the defined subgroup. Group selection in the left window may be done by using the *Control* and *Shift* buttons on the keyboard accompanied by the mouse. All the highlighted wells will move to the right window by selecting *INSERT*. This action will insert the *GROUP NAME* in the first available cell above the selected wells in the right window.
3. To form a second subgroup, repeat *Step 1*. Do not insert blank rows into the table in the right window. Please note that a particular well may be placed into more than one subgroup. To remove a well or group of wells from a subgroup, highlight the well or wells in the right window and press *DELETE*. This will not delete the well altogether, but simply remove it from the subgroup. To clear the entire right window of all data and subgroups press *CLEAR ALL*.

6.3 CALCULATE SUBGROUPS

The developed subgroups from the *MARK SUBGROUPS* screen are displayed in tables on two separate tabs on the *CALCULATE SUBGROUPS* screen.

1. The values shown in the table on the left tab (Fig. 31) are the calculated areas for each time point of each well for the groups of processed images from the *MARK SUBGROUPS* screen.
2. The values shown in the table on the right tab (Fig. 32) are the calculated subgroup average areas per time step for each subgroup.
3. The user may choose to *EXPORT TO EXCEL (.csv)* or *EXCEL TO TEXT (.txt)*. The *EXPORT TO EXCEL* action creates a *Comma Separated Values file (.csv)*. Once this *(.csv)* file is saved to a designation, the file may be resaved or reopened and saved as a Microsoft Excel file or opened in other data analysis software programs.

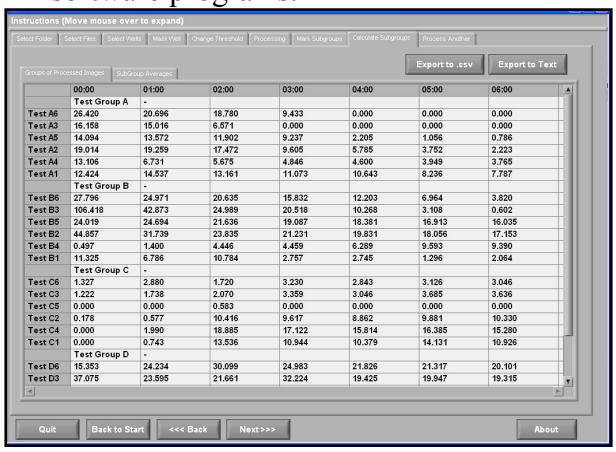

Figure 31. *Creating subgroups with the wells.*

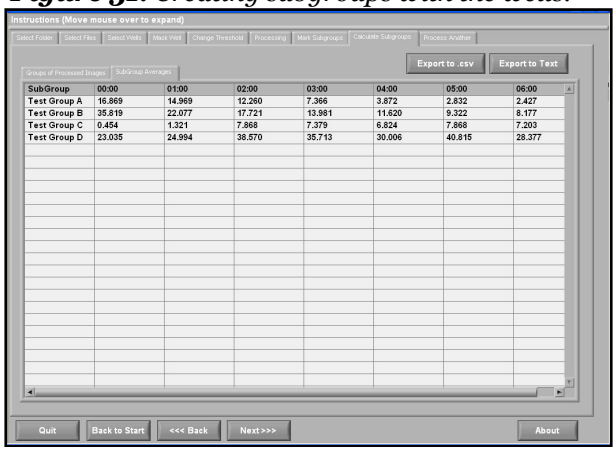

Figure 32. *Display of the subgroup averages.*

APPENDIX

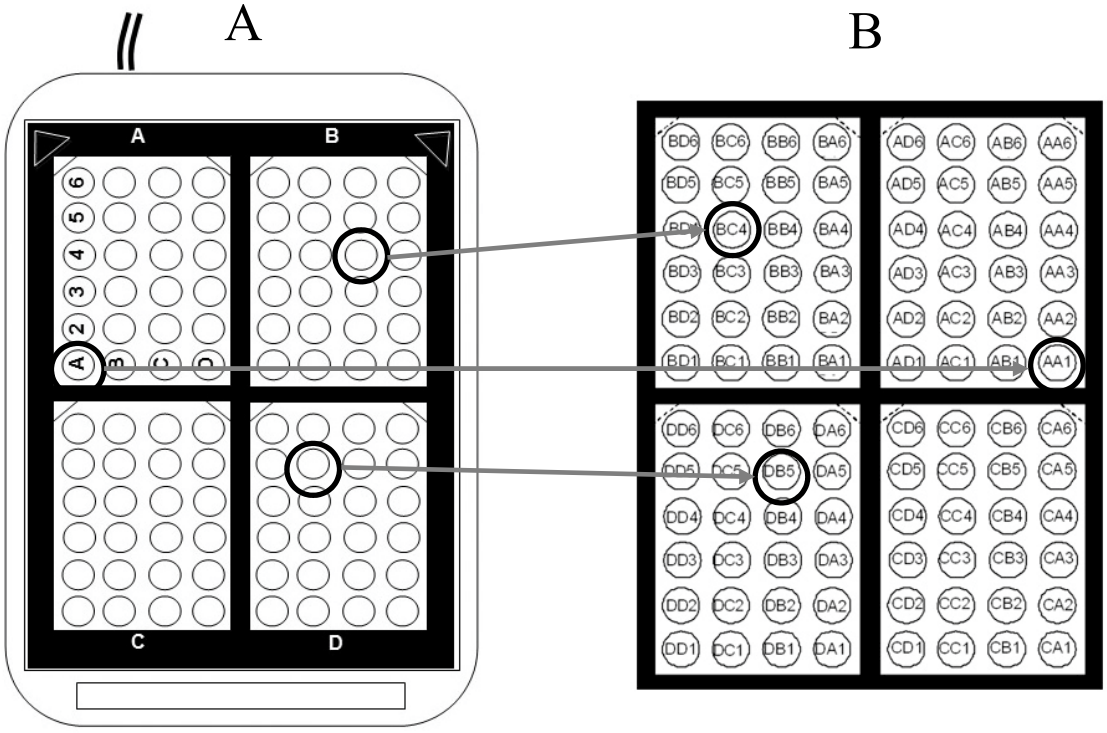

Figure 33. *Physical orientation of the wells in Flexcell's 24-well plates on the scanner bed (A) and their corresponding locations in the scanned image (B).*

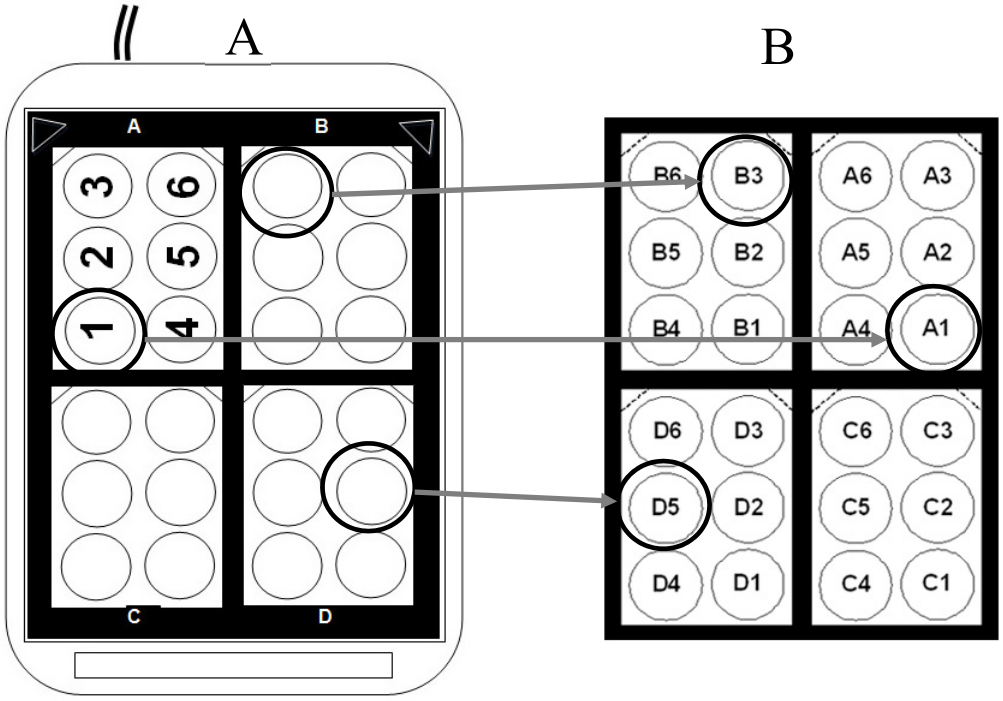

Figure 34. *Physical orientation of the wells in Flexcell's 6-well plates on the scanner bed (A) and their corresponding locations in the scanned image (B).*

Table 2. Relationship between image file size and image parameter setting.

Image file type	Resolution setting (dpi)	Quality setting (%)	Image size (MB)
BMP	600	N/A	102
JPG	600	100	19.2
		75	2.96
		50	1.96
		25	1.29
BMP	300	N/A	25.6
JPG	300	100	5.53
		75	0.93
		50	0.62
		25	0.39
BMP	150	N/A	6.4
JPG	150	100	1.44
		75	0.28
		50	0.19
		25	0.13