

Quantitative Analysis of Clot Deposition on Extracorporeal Life Support Membrane Oxygenators Using Digital and Scanning Electron Microscopy Imaging Techniques

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Abstract

Device-induced thrombosis remains a major complication of extracorporeal life support (ECLS). To more thoroughly understand how blood components interact with the artificial surfaces of ECLS circuit components, assessment of clot deposition on these surfaces following clinical use is urgently needed. Scanning electron microscopy (SEM), which produces high-resolution images at nanoscale level, allows visualization and characterization of thrombotic deposits on ECLS circuitry. However, methodologies to increase the quantifiability of SEM analysis of ECLS circuit components have yet to be applied clinically. To address these issues, we developed a protocol to quantify clot deposition on ECLS membrane oxygenator gas transfer fiber sheets through digital and SEM imaging techniques. In this study, ECLS membrane oxygenator fiber sheets were obtained, fixed, and imaged after use. Following a standardized process, the percentage of clot deposition on both digital images and SEM images was quantified using ImageJ through blind reviews. The interrater reliability of quantitative analysis among reviewers was evaluated. Although this protocol focused on the analysis of ECLS membrane oxygenators, it is also adaptable to other components of the ECLS circuits such as catheters and tubing.

Key features

- Quantitative analysis of clot deposition using digital and scanning electron microscopy (SEM) techniques
- High-resolution images at nanoscale level
- Extracorporeal life support (ECLS) devices
- Membrane oxygenators
- Blood-contacting surfaces

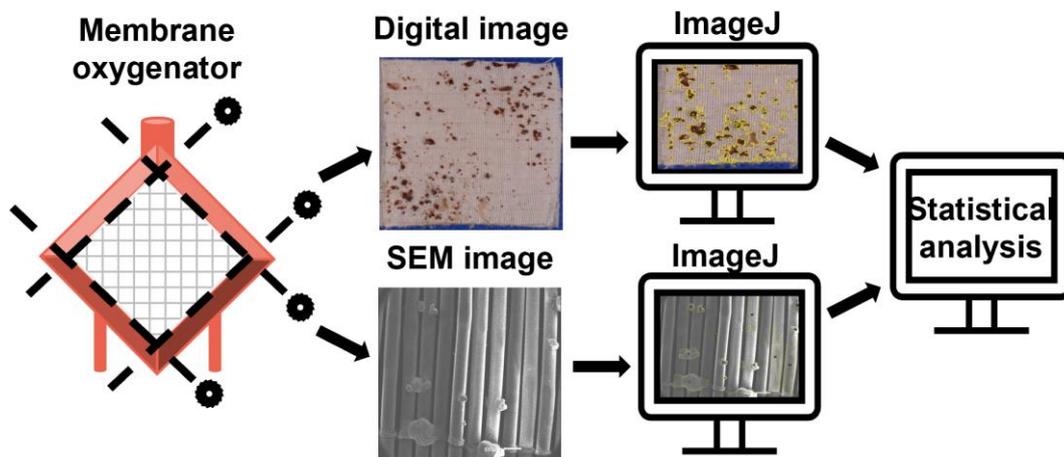
Keywords: Quantitative analysis, Scanning electron microscopy, ImageJ, Membrane oxygenator, Extracorporeal life support (ECLS), Clot deposition, Thrombosis

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Graphical overview



Background

Device-induced thrombosis remains one of the major complications of extracorporeal life support (ECLS) (Jaffer et al., 2015; Doyle and Hunt, 2018). The interaction of blood and artificial surfaces of ECLS components results in clot deposition on these surfaces and in severe instances can lead to occlusion of the circuit and systemic complications (Roberts et al., 2020a). Clinically available antithrombotic treatments can introduce hemorrhagic complications, especially in patients with preexisting bleeding disorders (MacLaren et al., 2022). Non-thrombogenic circuits that reduce thrombotic complications and enable improved patient outcomes are needed. An important prerequisite to developing such circuits is to more thoroughly understand how blood components interact with the artificial surfaces of ECLS devices (Beely et al., 2016). However, there is currently no methodology for routine examination of these devices after use. Surfaces of ECLS components could be specifically engineered to address the issue of thrombosis with a better understanding of the interactions between blood and ECLS surfaces. Examination and analysis of extracorporeal circuitry following clinical use in a standardized and validated method could inform both clinical decision-making regarding anticoagulation as well as device design.

Digital images have been used to record and visualize the extent of clot formation within ECLS devices in small pilot research studies, both clinically and in translational research laboratories (Lehle et al., 2008; Beely et al., 2016; Diehl and Gantner, 2018; Chlebowski et al., 2020; Naito et al., 2021). Blood clots are easily observed through digital images, while some microscopic thrombotic structures are not. Scanning electron microscopy (SEM), an advanced technology, produces high-resolution images at nanoscale level. Although most of the clinical use of SEM is qualitative, new methodologies that increase the quantifiability of SEM have been developed (Kundu et al., 1988; Di Iorio et al., 2005). We have previously used SEM to visualize and characterize clots and cell deposits in ECLS circuits following use in translational research studies in swine up to 72 h in duration (Beely et al., 2016; Roberts et al., 2020b). In this protocol, we developed and standardized a method to quantitatively analyze clot deposition using both digital and SEM images, allowing to routinely evaluate ECLS components following clinical use. Although this protocol focused on the analysis of ECLS membrane oxygenators only, this method could be applied to other components of the ECLS with necessary adaptations.

Materials and reagents

Reagents

1. Glutaraldehyde, 50% biological grade (Electron Microscopy Sciences, catalog number: 16520)
2. Sucrose, $\geq 99.5\%$ (Sigma-Aldrich, catalog number: S9378)
3. Sodium cacodylate trihydrate, $\geq 98\%$ (Sigma-Aldrich, catalog number: C0250)
4. Phosphate buffered saline (PBS) powder, 10 \times , pH 7.4 (Fisher BioReagents, catalog number: BP665-1)
5. Dehydrant alcohol, 100% (Epredia, catalog number: 6215)
6. Milli-Q water, 18.2 M Ω ·cm, obtained through Barnstead™ Smart2Pure™ Pro Water Purification System

Solutions

1. Scanning electron microscopy fixative (SEM fixative) (see Recipes)
2. Scanning electron microscopy buffer (SEM buffer) (see Recipes)
3. Phosphate buffered saline, pH 7.4 (PBS) (see Recipes)

Recipes

1. SEM fixative

Reagent	Final concentration	Quantity
Sucrose	0.1 M	8.5575 g
Sodium cacodylate trihydrate	0.1 M	5.3508 g
Glutaraldehyde, 50%	3%	15 mL
Milli-Q water	n/a	235 mL

2. SEM buffer

Reagent	Final concentration	Quantity
Sucrose	0.1 M	8.5575 g
Sodium cacodylate trihydrate	0.1 M	5.3508 g
Milli-Q water	n/a	250 mL

3. PBS (based on vendor instruction)

Reagent	Final concentration	Quantity
PBS powder, 10 \times	1 \times	98.9 g
Milli-Q water	n/a	10 L

Laboratory supplies

1. Slotted tissue cassette (Epredia, catalog number: B851729WH)
2. Container, multi-purpose, PP, with separate snap lid, 120 mL (Globe Scientific, catalog number: 271004)
3. Copper conductive tape, single adhesive surface (Ted Pella, catalog number: 16072-1)
4. Carbon conductive tape, double coated (Ted Pella, catalog number: 16073-5)
5. T10 Torx Screwdriver (Tekton, model number: DST31100)
6. Sterilization wrap (Halyard, catalog number: 34162)
7. Premium blue indicating silica gel desiccant beads 3–5 mm (Dry & Dry, catalog number: X000TS11ZP)
8. Straight connector, female luer lock port (Qosina, catalog number: 73317)
9. Needleless injection site, swappable, male luer lock (Qosina, catalog number: 80147)
10. 50 mL syringe, luer-lok tip (BD, catalog number: 309653)
11. Scissors

12. Surgical grade stainless steel forceps, nonlocking thumb handle straight delicate, serrated tips, 4 3/4" in. (McKesson, catalog number: 43-1-772)
13. Pathology metric ruler
14. Weighing paper or weighing boat
15. Spatula
16. Cylinder
17. Desiccator, 6"
18. SEM fixative/buffer waste bottle
19. Dehydration/ethanol waste bottle
20. Labels with study ID, protocol number, and date
21. Personal protective equipment (gloves, goggles, hearing protection, and scrubs/lab coats)

Equipment

1. Miter saw coupled with diamond blade (Miter saw DeWALT, model: DWS779; blade RIDGID, model: 12 in. dual-purpose)
2. Digital camera with lens (Nikon, camera model: D3300, lens model: AF-P DX NIKKOR 18-55 f/3.5-5.6G VR)
3. Barnstead™ Smart2Pure™ Pro Water Purification System (Thermo Fisher Scientific, catalog number: 50157873)
4. Gold sputter coater (Denton Vacuum, model: Desk II)
5. InTouchScope™ scanning electron microscope (JEOL, model: JSM-IT100)
6. Impact suction unit (Envi health solutions, model: 326/326M)

Software and datasets

1. ImageJ (version: 1.53k, release date: 07/06/2021, free)
2. Analytics Software & Solutions (SAS) (version 9.4, release date: 07/2013, license needed)

Procedure

A. Membrane oxygenator preservation post-use

1. Disconnect membrane oxygenator from ECLS system pump.
2. Flush membrane oxygenator from the inlet port with 1× PBS and drain blood/PBS flush from the outlet port until flush exiting outlet is clear (standardize flush flow rate to the flow rate in which the ECLS device is operated).
3. Connect inlet and outlet tubing (10–15 cm length) into a closed loop with 3/8" × 3/8" connector (Figure 1).
4. Attach needleless injection port to 3/8" × 3/8" straight connector.
5. Inject SEM fixative into the oxygenator via a priming port using a 50 mL syringe while simultaneously withdrawing air from the oxygenator via a separate port. This process is continued until the membrane is saturated with fixative.
6. Store the membrane oxygenator in the refrigerator (4 °C) until ready for section B.

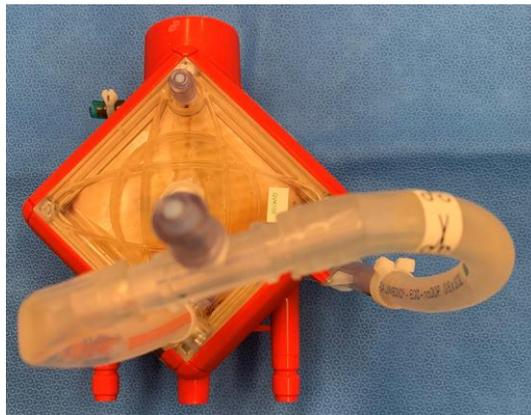


Figure 1. Membrane oxygenator preservation with SEM fixative

B. Membrane oxygenator disassembly (General note 1)

1. Drain SEM fixative slowly into SEM fixative/buffer waste bottle.
2. Rinse the membrane oxygenator with 500 mL of 1× PBS.
3. Unscrew and disassemble membrane oxygenator pump using a T10 Torx screwdriver.
4. Take digital images of membrane oxygenator pump, inlet face, and outlet face of the membrane oxygenator on sterilization wrap (use as a blue background) with labels and pathology metric ruler. Examples are in Figure 2.

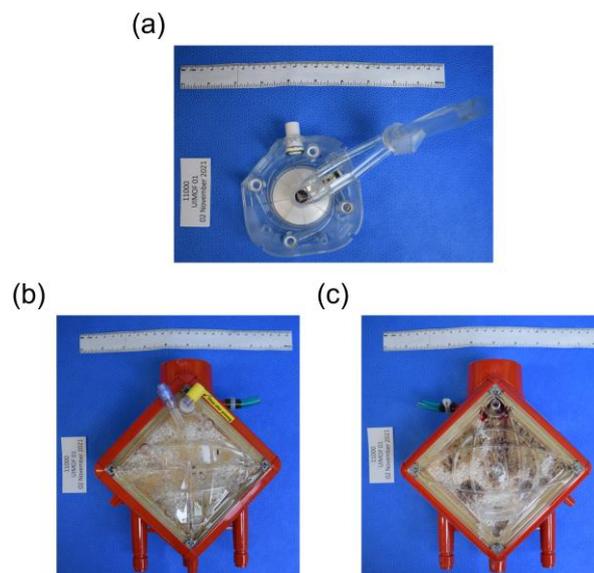


Figure 2. Example images of (a) membrane oxygenator pump, (b) inlet/top/pre-membrane/venous face of the membrane oxygenator, and (c) outlet/bottom/post-membrane/arterial face of the membrane oxygenator

5. Cut off water connections to get a flat edge, then cut off post-membrane sample port on outlet side so that the membrane can sit flat on the miter saw table (Figure 3).

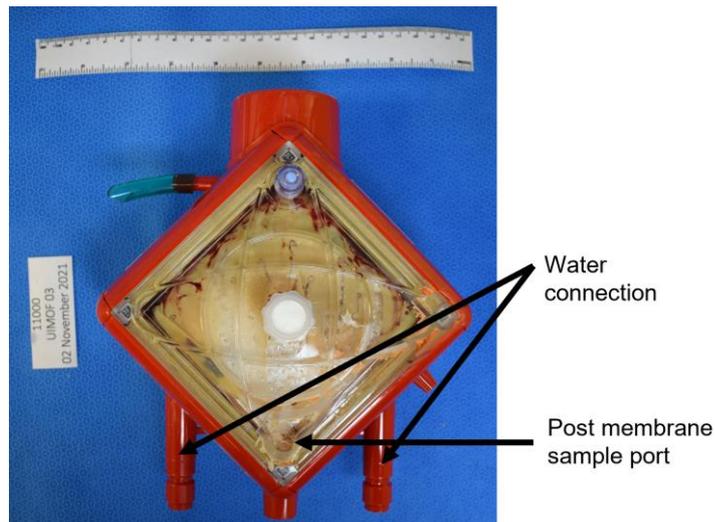


Figure 3. Examples of water connection and post-membrane sample port of a membrane oxygenator

6. Cut the four sides of the membrane case with inlet face facing up using a miter saw coupled with vacuum system to remove debris (schematic shown in Figure 4).

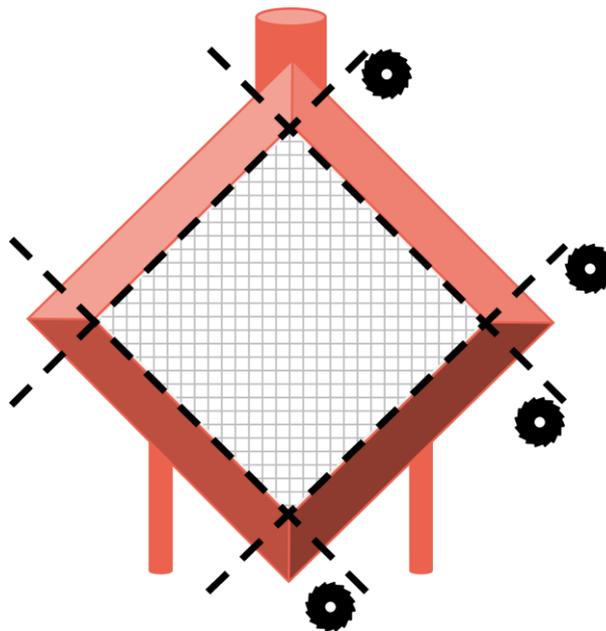


Figure 4. Schematic of membrane oxygenator dissection

7. Gently remove inlet face plastic cover using forceps.
8. The first gas transfer fiber sheet should be exposed and ready for imaging.

C. Membrane oxygenator gas transfer fiber sheet digital imaging (Troubleshooting 1)

1. Gently remove gas transfer fiber sheets individually in order from inlet face to outlet face.
2. Place every four gas fiber sheets next to each other horizontally with total three rows.
3. Take digital images of every 12 gas fiber sheets with Study ID and number labels as shown in Figure 5.



Figure 5. Layout example of membrane gas transfer fiber sheets for digital imaging

4. Take individual images of top inlet membrane gas fiber sheet, middle membrane gas fiber sheet, and bottom outlet membrane gas fiber sheet with study ID, date, and pathology metric ruler as shown in Figure 6.



Figure 6. Example of individual membrane gas fiber sheet for digital imaging

5. Cut top inlet, middle, and bottom outlet membrane gas transfer fiber sheets each into nine squares (total 27 squares). Number squares from top inlet membrane gas fiber sheet from 1 through 9, middle membrane gas fiber sheet from 10 through 18, and bottom outlet membrane gas fiber sheet from 19 through 27, as shown in Figure 7.

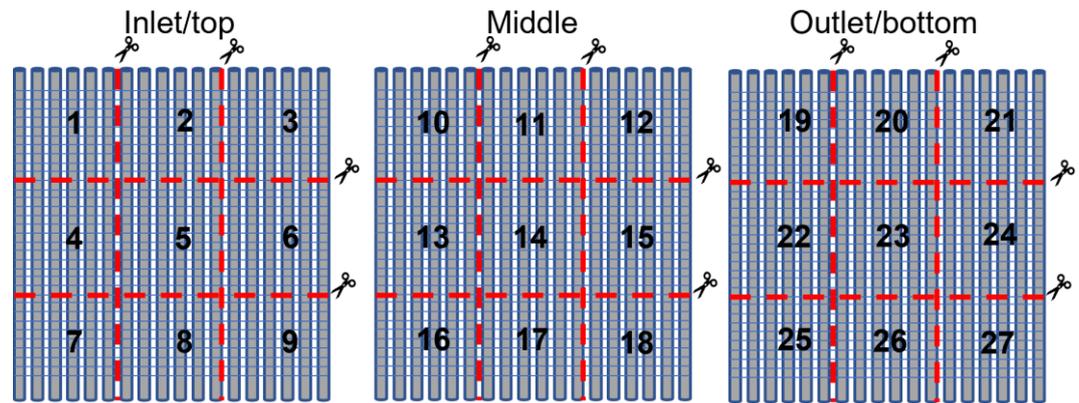


Figure 7. Schematic diagram of cutting individual membrane gas fiber sheets and numbering. Numbers 1–9 are assigned to inlet/top membrane gas fiber sheet (left), numbers 10–18 are assigned to middle membrane gas fiber sheet (middle), and numbers 19–27 are assigned to outlet/bottom membrane gas fiber sheet (right).

6. Place randomly selected squares into labeled cassettes with the surface that was closest to the membrane inlet facing the lid of the cassette facing upward.
7. Place cassettes with sample into 120 mL container, add SEM fixative, and store in refrigerator (4 °C) until ready for section D.

D. SEM sample dehydration (General Note 2)

1. Drain SEM fixative from each container into SEM fixative/buffer waste bottle.
2. Add SEM buffer into each container and incubate for 10 min; drain SEM buffer into SEM fixative/buffer waste bottle.
3. Add 30% dehydrant into each container and incubate for 10 min; drain 30% dehydrant into dehydrant/ethanol waste bottle.
4. Add 50% dehydrant into each container and incubate for 10 min; drain 50% dehydrant into dehydrant/ethanol waste bottle.
5. Add 70% dehydrant into each container and incubate for 10 min; drain 70% dehydrant into dehydrant/ethanol waste bottle.
6. Add 100% dehydrant into each container and incubate for 10 min; drain 100% dehydrant into dehydrant/ethanol waste bottle.
7. Add 100% dehydrant into each container and incubate for 10 min; drain 100% dehydrant into dehydrant/ethanol waste bottle.
8. Place container with samples into desiccator overnight under vacuum.
9. After overnight vacuum drying, fill 1/5 of the container with silica gel desiccant beads and close the lid tightly.
10. Keep the container with samples at room temperature until ready to do SEM imaging.

E. SEM imaging

1. SEM sample preparation (Figure 8)
 - a. Attach sample onto an SEM sample holder using double-sided conductive carbon tape.
 - b. Sputter coat with 20 nm of gold nanoparticles using Denton Vacuum Desk II Gold sputter coater.
 - c. Tape the opposite borders of the sample with conductive copper tape perpendicular to the sample fibers.

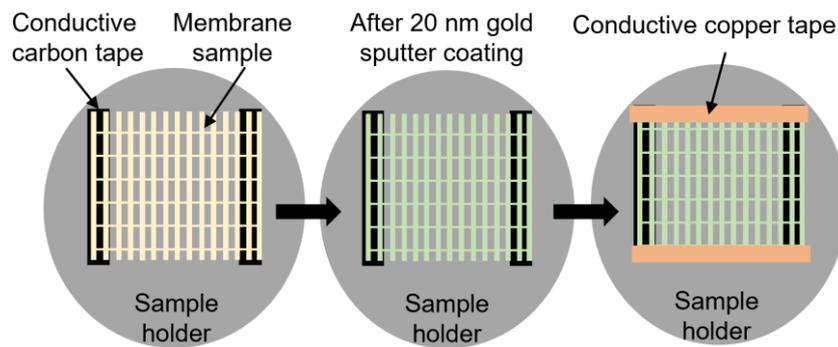


Figure 8. Schematic of SEM sample preparation process

2. SEM imaging for percentage of clot deposition quantitative analysis
 - a. Set up 14 kV accelerating voltage with 14 mm working distance using InTouchScope™ scanning electron microscope.
 - b. Take images at 25× magnification horizontally from the upper left corner of sample to the lower right corner to cover the entire surface of each sample.

F. Quantitative analysis of digital images using ImageJ

1. Open ImageJ software and open the image for analysis. Click *File* and select *Open* to open an image.
2. Set up scale of the image (Figure 9).
 - a. Click *Analyze* and select *Set Scale...*
 - b. Enter 0 next to *Known distance*.
 - c. Enter *pixels* next to *Unit of length*.

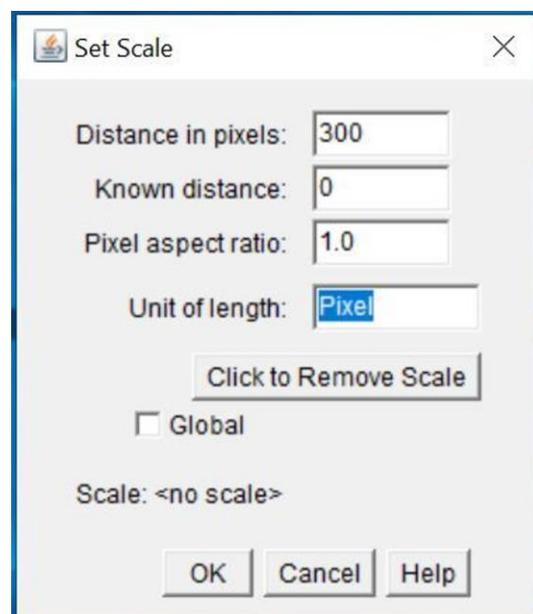


Figure 9. Screenshot of *Set Scale* window

3. Select total area of interest (Figure 10).
 - a. Click *Image*, *Adjust*, and then *Color Threshold...*
 - b. Set *Thresholding method* as *Default*.

- c. Set *Color space* as *HSB* to start and change to *RGB* if needed.
- d. Check *Dark background* box.
- e. Set threshold to cover the entire surface of the membrane gas fibers and exclude blue background.

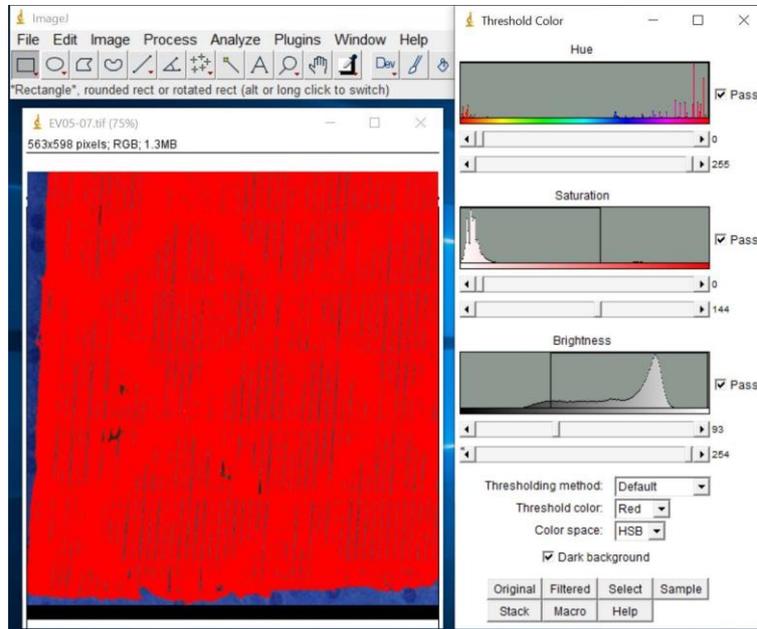


Figure 10. Screenshot after selecting total area of interest

- f. Click *Select* to highlight the total area of interest (Figure 11).

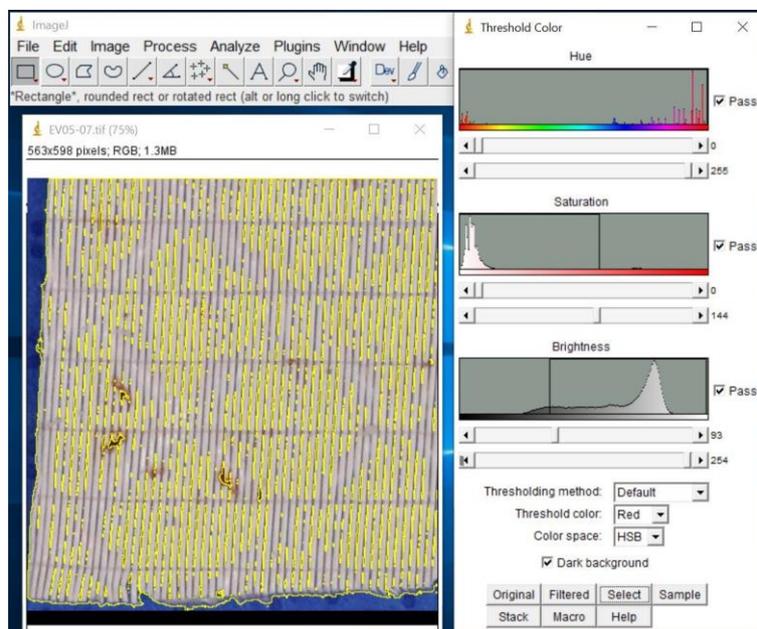


Figure 11. Screenshot of highlighted total area of interest after selection

- g. Click *Analyze* and select *Measure* to show the results in *Results* window (Figure 12).

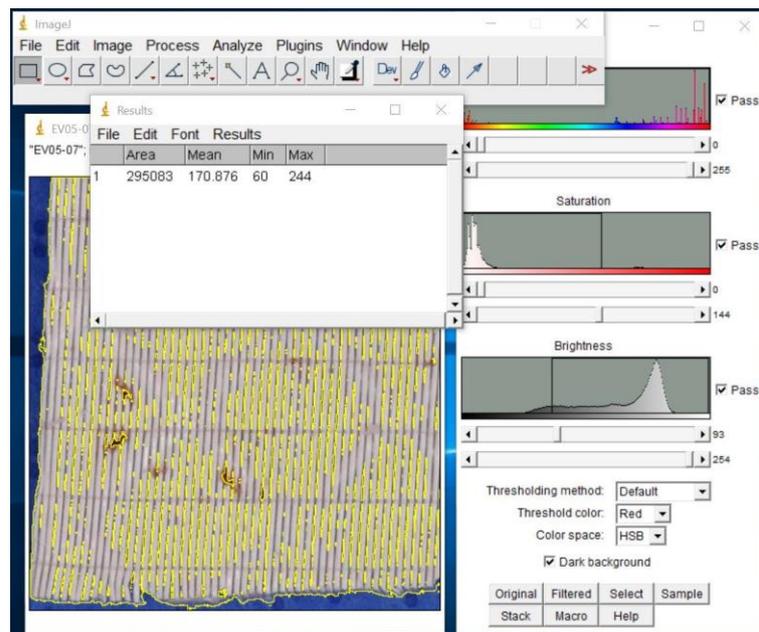


Figure 12. Screenshot of *Results* window

- h. Enter the Area value into the Excel spreadsheet as total area.
4. Select clot deposition area of interest (Figure 13).
 - a. Click *Original* on the *Threshold Color* window to revert to the original image.
 - b. Adjust threshold to cover clot deposition area only.

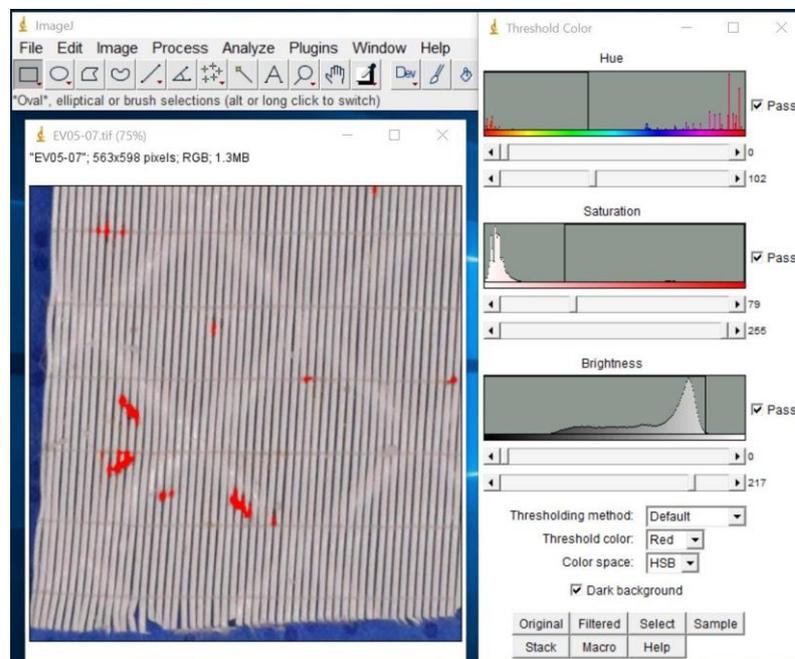


Figure 13. Screenshot after selecting clot deposition area of interest

- c. Click *Select* to highlight the clot deposition area.
- d. Click *Analyze* and select *Measure* to show the results in *Results* window.

- e. Enter the Area value into the spreadsheet as clot deposition area.
5. Calculate percentage of coverage using clot deposition area and total area on the spreadsheet (an example is shown below, Figure 14).

MOF EV05-3-25X									
Image	Reviewer 1			Reviewer 2			Reviewer 3		
	Selected Area	Total Area	% of Coverage	Selected Area	Total Area	% of Coverage	Selected Area	Total Area	% of Coverage
1	70543	1023854	6.8899472	140823	888641	15.84700683	176014	994957	17.69061377
2	324346	934714	34.7000259	263914	1068093	24.70889707	293905	1059302	27.74515672
3	272682	820428	33.2365546	119812	969519	12.35788056	310016	959917	32.2961256
4	385964	965946	39.9570991	207333	1164412	17.805811	314175	1147748	27.37316902
5	47720	1065528	4.4785308	202187	1095570	18.45495952	216868	1090714	19.88312243
6	75007	836374	8.9681171	173245	853805	20.29093294	204826	855534	23.9413045
7	97206	1188141	8.1813522	80282	1228800	6.533365885	255835	1194447	21.41869836
8	235159	1182642	19.8842084	106910	1228800	8.700358073	295586	1194380	24.74807013
9	180643	1193659	15.1335515	197093	1228800	16.0394694	322557	1198647	26.91009113
10	346302	1181141	29.3192769	241412	1228800	19.64615885	515316	1198697	42.98967963
11	69741	1026010	6.7973022	120421	1019198	11.81527044	216726	1033690	20.96624713
12	75030	1180966	6.3532735	103795	1228800	8.446858724	115343	1178252	9.789331993
13	147311	1201631	12.2592543	133130	1228800	10.83414714	166686	1190596	14.00021502
14	139681	1195985	11.6791599	140632	1228800	11.44466146	178421	1194063	14.94234391
15	230385	1179756	19.5281906	287397	1228800	23.38842773	345896	1190949	29.04372899

Figure 14. Screenshot of a calculation spreadsheet example

G. Quantitative analysis of SEM images using ImageJ (General Note 3)

1. Open ImageJ software and open the image for analysis. Click *File* and select *Open* to open an image.
2. Set up scale of the image.
 - a. Click *Straight* line icon and draw a line on the scale bar (Figure 15).

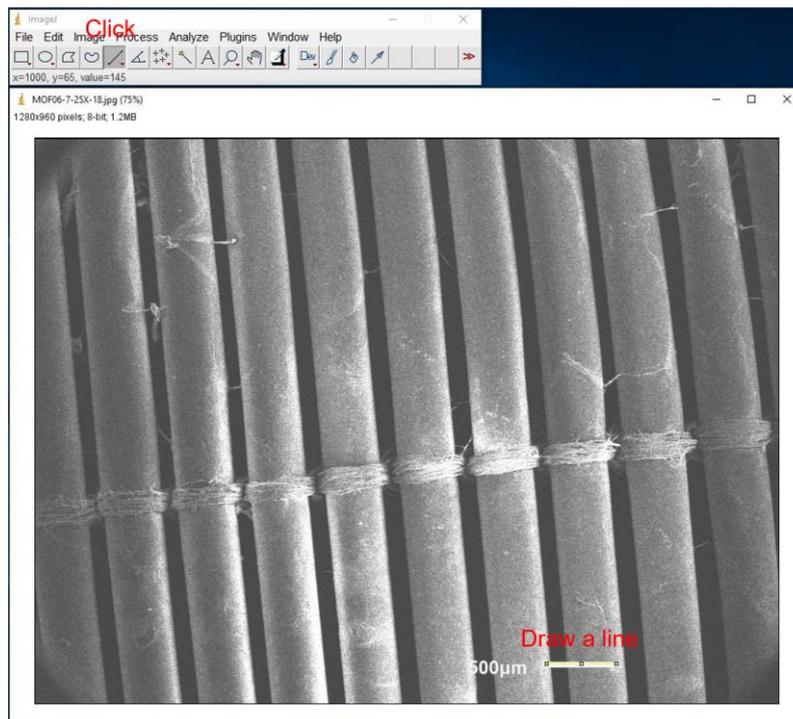


Figure 15. Screenshot of setting up scale for SEM images

- b. Click *Analyze* and select *Set Scale...* to change unit from pixel to micrometer (Figure 16).

- c. Enter scale bar value next to *Known distance* (Figure 16).
- d. Enter scale bar unit next to *Unit of length* (Figure 16).
- e. Click OK.

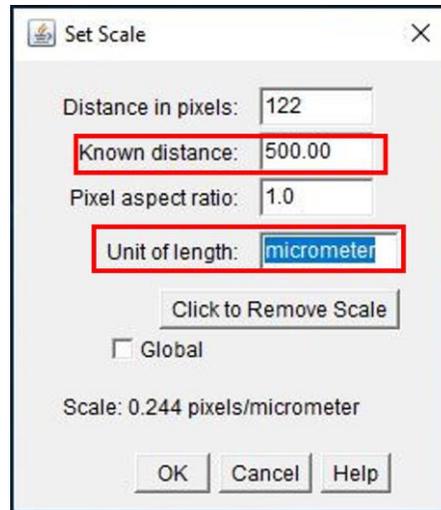


Figure 16. Screenshot of *Set Scale* window for SEM images

3. Click *Analyze* and then *Set Measurements*. Check boxes *Area* and *Display Label*. Click OK.
4. Click *Analyze, Tools*, and then *ROI Manager...* Check boxes *Show All* and *Labels*.
 - a. Use *Polygon selections* or *Freehand selections* to draw areas of interest (Figure 17).

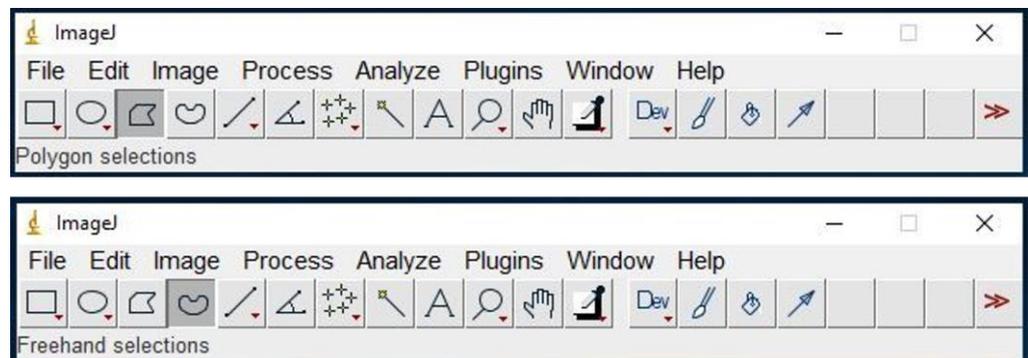


Figure 17. Screenshot of *Polygon* and *Freehand* selections

- b. After drawing area of interest, click *Add [t]* to add it to the list (same for all other areas of interest) (Figure 18).

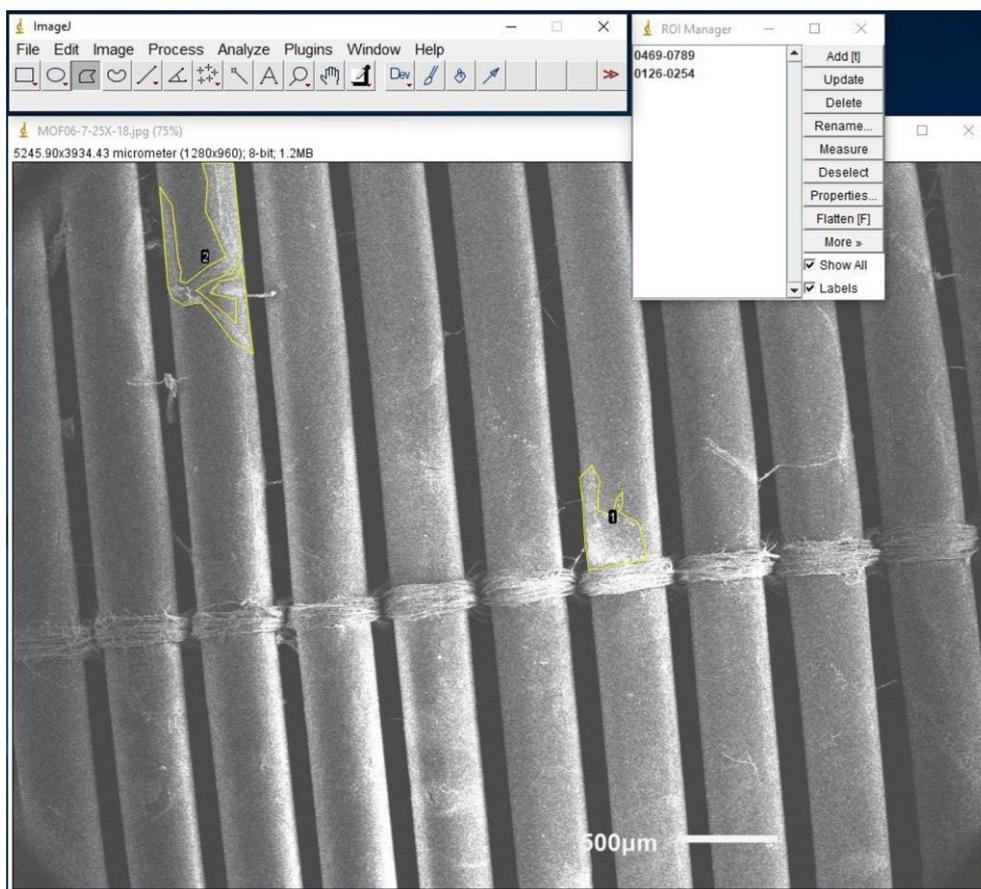


Figure 18. Screenshot of drawing area of interest example for SEM images

- c. Click one of the areas of interest. Click *Rename* and then enter the number of areas of interest next to *Rename As*. Click *OK* (same for all other areas of interest).
- d. Select all areas of interest, and then click *Measure*.
5. Save Results into .csv format. Click *File*, and then *Save As...*
6. Open saved file.
7. Use the value under Area column divided by total area (Figure 19), which is shown above each image in ImageJ to get percentage of coverage.

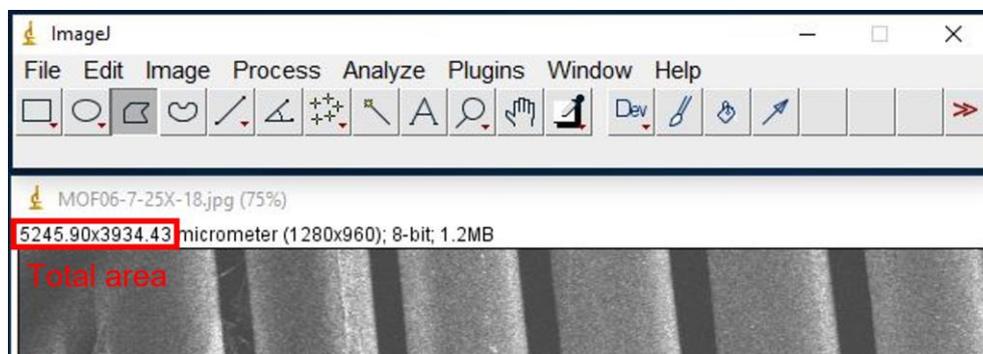


Figure 19. Screenshot of the location of total area

8. Screenshot and save image with areas of interest as for recorder cord.

Data analysis

Perform statistical comparison on digital and SEM images percentage of total clot coverage of each set of samples (two-sided test with $\alpha = 0.05$ for significance via SAS 9.4). Perform interrater reliability of quantitative analysis to evaluate the agreement among reviewers (General note 4).

Validation of protocol

Currently, there is no *gold standard* method for determining total clot deposition on medical device surfaces, therefore limiting the ability to compare our methods tested to an established methodology. This protocol has been validated through a blind review process in which individuals that are not familiar with this method were required to follow the protocol and perform the analysis without help. By the end of the process, we collected feedback and improved the protocol. So far, at least six individuals from three institutions have tried this protocol and there are no outstanding concerns or issues. In addition, this protocol results in a podium presentation at 37th Annual Children's National Symposium: ECMO & the Advanced Therapies for Respiratory Failure (Virtual).

Zang et al. (2021). Quantitative Analysis of Digital vs. Scanning Electron Microscopy Images as An Assessment Tool for Post-Extracorporeal Life Support Clot Formation Evaluation. Oral Presentation at Bradley Hill Best Abstract Session.

General notes and troubleshooting

General notes

1. These steps only apply to Maquet HLS7.0 membrane and may require modifications for other membrane oxygenators.
2. It is recommended to perform SEM imaging the day after SEM dehydration to avoid sample moisturization during storage. If it is not practicable, we recommend checking and changing silica gel desiccant beads on a regular basis.
3. It took approximately 28 h to image and process a set of SEM images for one sample compared to approximately 3 min for one digital image of the same sample.
4. To avoid bias, we suggest choosing individuals who are not familiar with the studies involved in membrane oxygenator or de-identified samples before performing the analysis. For routine post-study analysis, interrater reliability score is used to see the reliability and validity to ensure the results are accurate and replicable.
5. Hazardous exposure (e.g., blood, hazardous chemicals in fixative, etc.) that could occur when assessing materials after clinical use may raise safety and health concerns.
6. SEM is expensive and time consuming, especially at institutions where SEM is not available or there are not enough trained technicians to perform SEM on a routine basis. Digital imaging could be an alternative approach to access clot deposition; however, some nanoscale clots will be missing from digital images. Therefore, we recommend the researchers and clinicians to balance the advantages and disadvantages of these methods and make a conscious decision based on their needs.
7. The most challenging part during the development of this protocol is section B. We highly recommend trying at least one *test* membrane oxygenator before performing on the membrane oxygenator to be analyzed.

Troubleshooting

1. Some of the thrombi were noticed to be stripped from the sample surface during sample processing and handling as the thrombus did not adhere to the surface tightly. This may lead to variability in quantitative analysis vs. the clinical state of the device during use. Please be as gentle as possible in this step.

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Competing interests

There are no conflicts of interest.

Ethical considerations

Membrane oxygenators were obtained from animal studies that were carried out in compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council at the Autonomous Reanimation and Evacuation Research Program (AREVA) (San Antonio, TX). The University of Texas at San Antonio Institutional Animal Care and Use (UTSA IACUC) approved all research conducted (Protocol SU001-03-23). The AREVA laboratory facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The protocol and facility were also approved by the Department of Defense, Medical Research and Development Command Animal Care and Use Review Office.

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