Measurement Assurance Case Study: 
Nanofiber Diameter

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“Validatable” not “validated”, since validation is a continuous process that must be established by each lab that implements the measurement.

- **Measurement Assurance**: Evaluating & reducing variability in a measurement to improve confidence in results for use in decision-making
- Underpins development of documentary standards
• Measurement assurance strategies for measuring nanofiber diameter

• 2013 ASTM Workshop on Scaffold Standards & Measurements (Indianapolis, IN, USA): #1 need identified was “better measurements for scaffold structure”
DiameterJ: Automated Image Analysis

- Current practice is manual measurement using a line tool in imaging software (ImageJ)
  - Slow (10 min/image) & low n
  - Human bias

Increasing “n” (number of measurements) enables better statistics & better modeling of the probability distribution function (histogram)

Automation increases the number of measurements & reduces human bias
**Reference Materials** are homogeneous & stable in regard to specified properties for use in calibration, to serve as a control or to serve as a reference point for comparability (ISO Guide 35)
Steel Wire with Known Diameter

- Narrow gauge stainless steel wire (HSM Wire)
- Manufacturer measures dia. with resistivity & calipers
- Wire dia. verified with light microscopy & human manual segmentation in SEM

<table>
<thead>
<tr>
<th>Wire Gauge</th>
<th>Manufacturer Reported Dia. (µm)</th>
<th>Light Microscopy Dia. (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>31.0</td>
<td>31.1 (0.1)</td>
</tr>
<tr>
<td>50</td>
<td>25.5</td>
<td>25.6 (0.1)</td>
</tr>
<tr>
<td>53</td>
<td>16.75</td>
<td>16.7 (0.1)</td>
</tr>
</tbody>
</table>
Steel Wire with Known Diameter

48 Gauge Steel Wire

Frequency

Wire Diameter (μm)

0 10 20 30 40 50 60 70 80

0 2000 4000 6000 8000 10000 12000 14000 16000

31.44
**Flow Diagram** is a tool to formally map a measurement process so that each step can be considered for its contribution to measurement uncertainty.

**Measurement Process Flow Diagram**

1. Electrospin Fibers (Sample Preparation)
2. Image in Scanning Electron Microscope
3. Segment Images
4. Measure Fiber Diameter in Segmented Images
5. Analyze Data, Determine Fiber Diameter Distribution

**Steel Reference Wire**

**Synthetic Images**

**Graph:**
- X-axis: Fiber Diameter (µm)
- Y-axis: Frequency
- Data points for fiber diameter distribution

**Images:**
- Fiber images
- Measurement setup

**NIST**

**Material Measurement Laboratory**
Orthogonal Measurements:

Confidence in a measurement result is enhanced when multiple measurement methods give a similar value of a material property.

- Orthogonal Measurements
  - More precise than the measurement that you are trying to assure (slower, expensive, harder)
  - Based on a different physical principle
- Synthetic images
  - Counted pixels by hand (very IMPORTANT, MSPaint didn’t work)
- Steel reference wires
  - Manufacturer measured resistivity
  - Manufacture measured with calipers
  - Optical imaging of fibers
  - Human manual measurement with ImageJ line tool in SEMs
- Electrospun polymer fibers
  - Human manual measurement with ImageJ line tool in SEMs
Sensitivity Testing (Ruggedness Testing) (Design of Experiments)

Sensitivity Testing can identify key measurement parameters that must be controlled to make the measurement more reliable.

103 synthetic images:
- Different diameters
- Straight vs curved
- Aligned vs disordered
- Multiple diameters

Multimodal Diameter Samples

2 Diameters

6 Diameters

(Failed on 10 Diameters)
Establishing Fiber Diameter Measurement Range

Too small = too few pixels per fiber

Too big = not enough fibers per image, edge effects

Sensitivity Testing (Design of Exps.)

Dia. = 3 px

Dia. = 250 px

Percent Error

Line Diameter (Pixels)
Effect of Segmentation (24 Algorithms Tested)
Effect of Segmentation on DiameterJ Results

Raw SEM micrograph of 53 ga, steel reference wire (fiber dia. 16.7 µm)

Sensitivity Testing (Design of Exps.)

Global Otsu, Global Min Error, Machine Learning, Local Otsu
Process Controls are procedures to monitor critical points in a measurement process to check that steps are performing according to specifications.

Visually compare raw image with:
- Segmentations
- Euclidian distance map
- Histogram (bimodal?)
- DiameterJ results

Manufacturer’s Fiber Dia. = 31.0 µm

<table>
<thead>
<tr>
<th>DiameterJ Results</th>
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<tbody>
<tr>
<td>Mean</td>
<td>31 µm</td>
</tr>
<tr>
<td>Mode</td>
<td>32 µm</td>
</tr>
<tr>
<td>Median</td>
<td>32 µm</td>
</tr>
</tbody>
</table>
• To help identify errors, DiameterJ has a locator tool which labels locations where fibers diameters of a given range were found.

• Fibers along image edge, poor segmentation or fiber overlap can yield errant measurements.

Red Lines = 1 px to 255 px

Red Lines = 40 px to 255 px
Performance Specifications

- Fibers must be at least 10 px in diameter
- Fibers should not be greater than 10% of the smallest dimension of the image
  - Example: SEM imaging of 500 nm fibers should be conducted at a magnification between 1500X and 10000X for a 1280 px by 960 px image capture
- Visual Examination: Fiber diameters in raw images qualitatively agree with segmentations & DiameterJ results
- For multimodal distributions, modes must be separated by more than 3 px
- In the system tested, 6 fiber dia. peaks is maximum # of peaks for 1 image
- If you don’t meet these specifications…then test result should be questioned (possibly rejected)

Performance Specifications are established by the user from sensitivity testing & charting process control data; if test specifications are not met, then results can not be used in decision-making.
Web Training Module

Operator Training

Operator Training improves measurement precision to improve comparability between different operators & labs.

Web training where users download & analyze images with DiameterJ.

Understanding Output from DiameterJ

Fill out the below information and begin the 20 question Quiz only AFTER reading the documents found at the links below.

* Required

- Participant Number *
  - Save this number somewhere: DO NOT LOSE THIS NUMBER. You will need it later.
  - 953128

- Installation and Use of DiameterJ - [https://goo.gl/4eS0P](https://goo.gl/4eS0P)
- Output of DiameterJ - [https://goo.gl/1erUc](https://goo.gl/1erUc)
Comparing Operator Performance
Before & After Training

• **IN PROGRESS:** Intra-lab comparability with Matt Becker Lab (Univ. of Akron, USA), 17 students analyzing images before/after training to assess improvement

• Test images of reference wires (48 ga. & 50 ga.)

• **Protocol Refinement:** Keep magnification constant

**Inter-Laboratory Comparisons** assess the robustness of an assay across different labs & results are used to refine the protocol
Ishikawa Diagram is a graphical tool to identify potential sources of variability in a process.

- Developed in the 1960s by Kaoru Ishikawa, who pioneered quality management processes in the Kawasaki shipyards.

Ishikawa Diagram (Cause & Effect)

- Sample Properties:
  - Fiber Size Relative to Image Size (Magnification)

- Image Capture:
  - 2° Electron Emission
  - Charging
  - Sputter Coat
  - Operator Skill

- Instrument Settings:
  - Voltage
  - Current
  - Working Distance
  - Vacuum Pressure
  - Gas in Chamber
  - Instrument Model
  - Operator Skill

- Automated Analysis with Algorithm:
  - Segmentation Algorithm (for Automated Analysis)

- Image Analysis:
  - Mean vs Mode vs Peak Fitting

- Operator Doing Manual Line Tool Analysis

- Data Analysis

Image Capture

Variability in a Fiber Diameter Measurement

Sample Properties

Cause

Effect
Comparing Operator Performance Before & After Training

Inter-Laboratory Comparisons assess the robustness of an assay across different labs & results are used to refine the protocol.

- **IN PROGRESS**: Intra-lab comparability study where participants analyze images before/after training to assess improvement.
- Test images are mixture of 48 ga. & 50 ga. Wire.
- **NEW SPECIFICATION**: Use constant magnification.

**Before Training**: 2 of 4 operators identified the bimodal distribution.

**After Training**: 4 of 4 operators identified the bimodal distribution.

![Graph showing fiber diameter measurements before and after training for 48 ga. and 50 ga. wires.](image)
Dissemination

Web:
• http://imagej.net/DiameterJ
• http://fiji.sc/DiameterJ
• https://github.com/NHotaling/DiameterJ

Papers:
• All Data & Images: Hotaling NA et al. (2015) Dataset for the validation and use of DiameterJ, an open source nanofiber diameter measurement tool. Data in Brief, in press.
Assuring Nanofiber Diameter Distribution Measurement

- Synthetic images: Counted pixels by hand
- Steel reference wires
  - Human manual measurement with ImageJ line tool
  - Optical imaging of fibers
  - Manufacturer resistivity measurement
  - Manufacturer caliper measurement
  - Electrospun PLGA fibers
  - Human manual measurement with ImageJ line tool

- “Intra”-lab underway (DiameterJ training module on the web)

- Compare raw image to:
  - Segmentations
  - Euclidian distance map
  - Histogram (bimodal?)
  - DiameterJ results
  - Identify errors w/ locator tool

- Fibers >10 px in dia.
- Fibers < 10% of the smallest image dimension
- Fiber dia. in raw images qualitatively agree with DiameterJ results
- Segmentations are of good quality when visually compared to raw image
- Multimodes separated by >3 px
- 6 fiber dia. peaks is maximum

- Fiber geometry
- Fiber dia. range
- # of modes
Summary

• “product consistency & lack of standards is possibly the single greatest challenge facing the field”

• Approach measurement process as a manufacturing process

• Measurement Assurance: Evaluate & reduce variability in order to improve confidence results to support decision-making (before writing a standard)

Thank you!