An Investigation of Process Parameters to Optimize the Fiber Diameter of Electrospun Vascular Scaffolds through Experimental Design

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Introduction

Electrospinning is a process used to produce fibers on the micrometer to nanometer scale from charged polymer solution. The electrostatic forces and the evaporation of the solvent as it travels from the ejector to the ground collector stretch the fiber continuously (1).

An in-house electrospinning device was built by Cal Poly to produce synthetic tissue engineered vascular grafts, which could be used for *in vitro* testing of intravascular devices (2). The electrospinning device essentially consists of a high-voltage power supply, microinjection pump, and a ground receptor. In order to develop the electrospun scaffolds for practical applications, it is important that a full understanding of the process parameters involved is achieved. The effects on the polymer output and the optimization of its fiber diameter will be the primary focus of this article. The four main factors affecting scaffold fiber diameter include solution concentration, applied voltage, gap distance, and flow rate because they are believed to have the greatest influence on the electrospinning process while being the simplest to control (2, 3). In the past, users have altered parameters in ways that they felt would allow for even, continuous fibers to be produced, however due to the lack of formal procedural empirical testing, few significant conclusions can be made from the current data.

From previous publications (2, 3, 4, 5) and the physical properties of the electrospinning process, theoretical predictions can be made about how changing the different parameters in a certain direction will affect fiber diameter:

Applied Voltage

Increasing the applied voltage would discharge the polymer jet with greater electrostatic repulsion, causing it to undergo higher levels of drawing stress. This

would result in a decrease in fiber diameter however, simultaneously, the fiber diameter distribution would become increasingly broader, making the control of the process more difficult.

Solution Concentration

A solution concentration below a certain threshold value will result in drops instead of fibers. High solution concentrations result in solutions with high viscosities, which could lead to processing problems (polymer solution flow to the needle tip becomes difficult to control, cohesive nature of the viscoelastic solution resists jet elongation)

There is a power law relationship between resulting fiber diameter and solution concentration. A higher solution concentration would give the liquid a higher viscosity, which resists jet elongation and thinning. This in turn would correlate to a larger fiber diameter produced. The value of the exponent is dependent on the polymer/solvent system used in the process (2).

Volumetric Flow Rate

Rate must be tuned so that a stable Taylor cone is formed. A low flow rate would form a vacuum in the needle, causing the Taylor cone to disappear and temporarily stop the electrospinning process. High flow rates could potentially cause a buildup of solution at the needle tip. As flow rate increases, the surface charge density decreases therefore the rate of charge withdrawal into the solution is dependent upon the residence time of ions in contact with the needle. At higher flow rates the solution spends less time in contact with the needle. It can be concluded that the

surface charge density is the driving force behind electrospinning, which is directly affected by flow rate.

Gap Distance

Gap distance is the distance from the charged Taylor cone to the collector and final fiber diameter. It follows a negative power relationship as increasing the distance allows bending instabilities and whipping action to elongate and decreases the diameter of the polymer jet. However, gap distances that are too great have negative results. There is a negative exponential relationship with surface charge density whereby increasing gap distance drops the surface charge density. As the distance between the charged solution and collector increases, the magnitude of the electric field between the two decreases, forming fewer charged ions (3,6).

Another process parameter is the diameter of the needle tip. Past investigations have suggested a lack of correlation between needle diameter used and resulting fiber diameter (7) in contrast to others that found fiber diameter to increase with a greater needle tip diameter (8, 9). Current investigators of the device have kept the needle diameter constant at 18G but further investigation of its effects on fiber diameter is recommended in the future.

Colby James, the initial investigator and designer of the device first investigated the electrospinning process using the polymer Poly(L-lactide-co-caprolactone) [P(LLA-CL)] in chloroform. Because these were the first experimental trials, among the preliminary fifteen runs in which certain parameters were changed, James came across multiple procedural obstacles affecting the accuracy of his data. Table 1 indicates the parameters that were used and changed between consecutive spins, noting specifically when errors occurred.

Run #	Altered Parameters	Results
1	Control (set by recommendations by Dr. Gene Boland (11)) [Solution] = 5.3% by weight P(LLA-CL) in chloroform Vol. Flow Rate = 6 ml/hr (3mL of sol.) 18 G needle Voltage = 16.5 kV GD = 10 in.	Bead-on fiber defects (discontinuous fiber diameters)
2	Decreased gap distance (**GD = 5 in**)	Flat fused surface from either electrospraying or fibers that did not reach bending instability phase causing solvent evaporation
3	Increased gap distance (**GD = 15 in**)	Bead-on-fiber defects
4	Decreased Voltage (**AV = 13 kV**) *switched to **27 G** needle	Bead defects present (decreased from previous spins)
5	Increased Voltage (**AV = 25 kV**)	*Voltage supply problem, process stopped immediately (no significant results to report)
6	Increased [solution] = **7.8% by weight P(LLA-CL) in chloroform**; amplifying power source with set voltage = 5 V at 1.5 A = 15 kV	Continuous fibers for the first time with high variation in diameter (<1 to 5 microns)
7	Increased [solution] **= 10.1% (since spin #6 resulted in continuous fibers)** *replaced needle to **18G** (AV = 15 kV channel set at 5 V, 1.5 A)	Continuous fibers with high variation in diameter
8	Increased [solution] = **9%** needle = 18G (Channel set at 5V 1.5 A = 15 kV)	Continuous fibers with observationally tighter interior surface distribution and decreased diameter than #6 & 7
9	Control parameters with External power source set at 5 V, 1.5 A in for AV	Bead-on-fiber defects on interior surface
10	Increased GD = **15 in**, AV **= 5V**, 1.5 A= 15 kV, [Solution] **= 7.8%**	Continuous, thinner fibers but less fiber collected on mandrel, resulting in fragile constructs
11	Vol. flow rate = **2ml/hr 27G** needle AV = **12 kV** GD = 15 in.	Bead-on-fiber defects
12	[sol] = **7.0%** by weight P(LLA-CL) in chloroform, AV = 15 kV	Bead-on-fiber defects
13	[sol] = **7.4%** by weight P(LLA-CL) in chloroform, AV = 15kV	Beads elongated on interior surface but still discontinuous fibers
14	[sol] = **7.6 %** by weight P(LLA-CL) in chloroform, AV = 15kV	Bead defects on interior surface
15	[sol] = **9.0 %** by weight P(LLA-CL) in chloroform, 5V, 1.5A = 15 kV	Continuous fibers similar to #8

Table 1: James' Parameter changes between spins and observations of resulting fibers (2)

Overall, James concluded that the solution concentration had the greatest effect on fiber diameter and as it increased, the larger the resulting fiber diameter was. However, below a certain concentration threshold (approximately 7% by weight 90:10 P(LLA-CL) in chloroform), the process becomes more like electrospraying. He also found that increasing the applied voltage, increasing the gap distance, and decreasing the needle diameter might

have decreased the average fiber diameter but did not have enough significant statistical evidence for any major conclusions (2).

The next investigator of the in-house device was Tiffany Pena who used the polymer Poly(D,L-lactide-co-glycolide) [PLGA] in chloroform. Using an 18 G needle, Pena used the first five spins to achieve a solution concentration that would produce continuous fibers and varied the flow rate and applied voltage accordingly. From her experimental methods, she decided to keep the solution concentration constant at 15 wt% PLGA in $CHCl_3$ and maintain a gap distance of 25.4 cm to investigate the effects of flow rate and applied voltage. Pena performed a two level, two factor factorial in which flow rate was tested at 3.0 ml/hr and 5.5 ml/hr in combination with applied voltage at 12.0 kV and 15.6 kV. Four treatments were run in total and results from the spins found that a 5.5 ml/hr flow rate at 12 kV yielded the most optimal fiber with an average mean of 5.49μm with a standard deviation of .93.

The current users of the device, Edward Siemsen and Yvette Castillo, have been exploring different parameters based on Pena's data and findings. They have kept the needle diameter at 18 G and from the most recent spins, have found a trend in increasing voltage. The most recent spins, their resulting average fiber diameter and standard deviations are in Table 2. This data shows a decreasing trend in fiber diameter as the applied voltage increases (Spins 1-4 & 7). Paired t-tests were performed between consecutive increases of voltage and although the increase in voltage between spin 2 and 3 and between spin 4 and 7 were insignificant there was a statistically significant trend between increasing voltage and resultant fiber diameter (Appendix A).

Spin	Voltage (kiloVolts)	Flowrate (mL/hour)	Gap Distance (inches)	Mean Fiber Size (microns)	St. Dev (s)	Data Points
1	15	5.5	10	3.73933	1.974638	261
2	18	5.5	10	3.39430	1.470551	288
3	21	5.5	10	3.427243	2.012853	288
4	24	5.5	10	2.142199	1.41993	297
5 *15% PLGA increase	18	5.5	10	2.64126	1.07858	288
6 *15% PLGA decrease	18	5.5	10	3.695292	4.87659 (globular formation)	287
7	27	5.5	10	2.018155	1.00063	296

Table 2: Spin data investigating voltage & solution concentration performed by Edward Siemens

Spin 5 and 6 were intended to test the effect of increasing and decreasing the solution concentration however at those parameters, decreasing the concentration seemed to be below the threshold of producing continuous fibers. Increasing the solution concentration in spin 5 seemed to decrease fiber diameter significantly, which is counterintuitive to the theoretical prediction (Appendix A). Further investigation in the effects of solution concentration on the produced fiber diameter is recommended as well as the effects of the other parameters that were held constant such as gap distance and flow rate.

Purpose of Project

The purpose of this report is to create a design of experiments for the electrospinning procedure that will be followed to allow an investigator to test the effectiveness of different strategies, thereby providing an opportunity for the improvement of the process through the reduction of common cause variation. That is, by understanding the current operation of the process and the factors that cause variation in the process outcomes, we can design an experiment by which we will understand more clearly the role that these potential factors

play in the variation of process outputs. Having statistical significance is necessary scientific evidence for the improvement of the process.

Fabrication

The electrospinning procedure that will be followed comes from previous tester, Tiffany Richelle Pena's thesis in 2009. (Appendix B) The investigator must follow these exact procedures for every spin that is performed. The only variation between spins should be the values at which the parameters are set.

Design of Experiment (DOE)

Any designed experiment has a few basic components and underlying concepts. The response variable represents the outcome that is measured and although it is possible to identify more than one, the main response variable for this report is the fiber diameter. The factors are variables that are deliberately changed for the expressed purpose of measuring the effect of the change of the response variable. In this experiment the factors will be fixed values because the electrospinning process only produces continuous fibers when parameter settings are at a specific range. The fixed factors are solution concentration, applied voltage, gap distance, and flow rate. A level of a factor is the specific condition of the factor at which we wish to observe the response variable. If more levels are to be examined, more treatments are required to be tested. A treatment is the set of conditions under which the response variable is to be observed. This experiment consists of several factors and one treatment is a specific combination of the different factor levels (10, pg 2-8).

For this experiment, a 2^f full factorial experiment with one center point is recommended due to its versatility and efficiency where the base, 2, indicates each factor to have two levels and f=4 as we have four factors (10, pg 140). A full factorial design includes every

treatment combination of factor levels possible and a center point would be an extra run including each factor at the midpoint between its two treatment levels. The purpose of the center point is to detect curvature in the fitted data. If there is curvature that involves the center of the design, the response at the center point will be either higher or lower than the fitted value of the factorial points.

The base values for the parameters come from analysis of the electrospinning device's previous tests. The most current runs being performed have used the polymer Poly (D, L-Lactide-co-glycolide) (PLGA) due to its ability to be electrospun into fibrous, porous constructs, and its ability to elicit appropriate cellular responses under physiological pulsatile flow. Tiffany Pena performed a solvent concentration analysis and concluded that a solution of 15 wt% PLGA in chloroform produced the optimal fibers. Currently, this solution concentration for testing has been used based on Tiffany's findings. The values of the testing variables for this design of experiment are based on the most recent electrospinning tests.

The recommended set of spins and parameters as stated previously are listed in Table 3 as a 4 factor, 2-level full factorial, with 1 center point and 2 replications. Two replications are required in an experiment in order to analyze interactions between factors. Minitab was used to generate the "Run Order" to ensure a randomized experiment rather than following the "Standard Order" which is the order in which the runs would be performed according to the Yates factorial analysis. Minitab created a DOE requiring 33 runs to be performed in order to observe all main and interaction effects (Appendix C). This is necessary because not all the parameter interactions have been analyzed thus far and the significant interactions must be determined in order to be able to run fewer runs with blocked insignificant interactions in future. The parameter values were chosen based on previous

data such that the difference between the 'high' and 'low' values would most likely have a statistical significance. The center point run is the middle value between each 'high' and 'low' parameter setting.

There is a possibility that not all spins will produce continuous fibers due to new parameter settings that have not previously been tested. If this is the case and the tester makes adjustments, they must be recorded and the real values of the parameters should be altered in the Minitab table. If any of the values must be altered, the experiment can still be analyzed using the same methods given, however Minitab will note that some factors might have more than two levels or that the design has some "botched" runs. The program will automatically analyze the experiment using a regression approach, which is also used to investigate and model the relationship between a response variable and its predictors.

StdOrder	RunOrder	CenterPt	Concentration	Voltage	Gap Distance	Flow Rate
2	1	1	15.0	24	7.0	4.5
8	2	1	15.0	30	10.0	4.5
5	3	1	10.0	24	10.0	4.5
17	4	1	10.0	24	7.0	4.5
28	5	1	15.0	30	7.0	6.5
20	6	1	15.0	30	7.0	4.5
24	7	1	15.0	30	10.0	4.5
27	8	1	10.0	30	7.0	6.5
21	9	1	10.0	24	10.0	4.5
6	10	1	15.0	24	10.0	4.5
25	11	1	10.0	24	7.0	6.5
26	12	1	15.0	24	7.0	6.5
14	13	1	15.0	24	10.0	6.5
23	14	1	10.0	30	10.0	4.5
3	15	1	10.0	30	7.0	4.5
22	16	1	15.0	24	10.0	4.5
31	17	1	10.0	30	10.0	6.5
13	18	1	10.0	24	10.0	6.5
29	19	1	10.0	24	10.0	6.5
1	20	1	10.0	24	7.0	4.5
33	21	0	12.5	27	8.5	5.5
18	22	1	15.0	24	7.0	4.5
15	23	1	10.0	30	10.0	6.5
12	24	1	15.0	30	7.0	6.5
7	25	1	10.0	30	10.0	4.5
16	26	1	15.0	30	10.0	6.5

19	27	1	10.0	30	7.0	4.5
32	28	1	15.0	30	10.0	6.5
4	29	1	15.0	30	7.0	4.5
10	30	1	15.0	24	7.0	6.5
11	31	1	10.0	30	7.0	6.5
9	32	1	10.0	24	7.0	6.5
30	33	1	15.0	24	10.0	6.5

Table 3: Design of Experiment to be Implemented (2^4 full factorial design with 1 Center Point & 2 replications)
StdOrder = Order of runs according to Yates Analysis
RunOrder = Order that spins should be run in (randomized by Minitab)
CenterPt: 1 = corner points, 0 = center point

Full Factorial Design

```
Factors:   4   Base Design:          4, 16
Runs:     17   Replicates:              1
Blocks:    1   Center pts (total):      1

All terms are free from aliasing.

Design Table (randomized)

Run  A  B  C  D
  1  +  −  −  +
  2  +  +  −  +
  3  −  +  −  −
  4  +  −  −  −
  5  −  −  −  +
  6  −  +  +  −
  7  +  +  +  +
  8  −  −  +  −
  9  −  +  −  +
 10  0  0  0  0
 11  +  −  +  −
 12  −  −  −  −
 13  +  −  +  +
 14  −  −  +  +
 15  +  +  +  −
 16  −  +  +  +
 17  +  +  −  −
```

Figure 1: Minitab Output for DOE
(+) = upper level treatment
(−) = lower level treatment
(0) = midpoint between treatment levels

Once all the runs in the experimental design are completed and fiber diameter measurements are collected, the mean fiber diameter for each treatment test should be calculated.

Analysis of Factorial Design

Using Minitab, an analysis of the experiments can be performed (Appendix C). The following steps can be done using the calculated output data:

1. Observational Method

   The response data can be plotted several ways to see if any trends or anomalies appear that would not be accounted for by the standard linear response model. Look at the distribution of all responses irrespective of the factor levels. A normal probability plot, a box plot, and a histogram of the response variable would be most appropriate (Figure 2). The normal probability plot of the residuals should follow a linear trend because this is an assumption that is made when making statistical conclusions. The histogram should have a bell-shaped curve, like a normal distribution would. In this case, the data is slightly skewed to the right. Next, responses versus Run Order can be examined to ensure there is no time sequence component affecting the response data. Next, plots of the responses sorted by factor columns should be made. It should be noted if plotted response data is very different between factor levels.

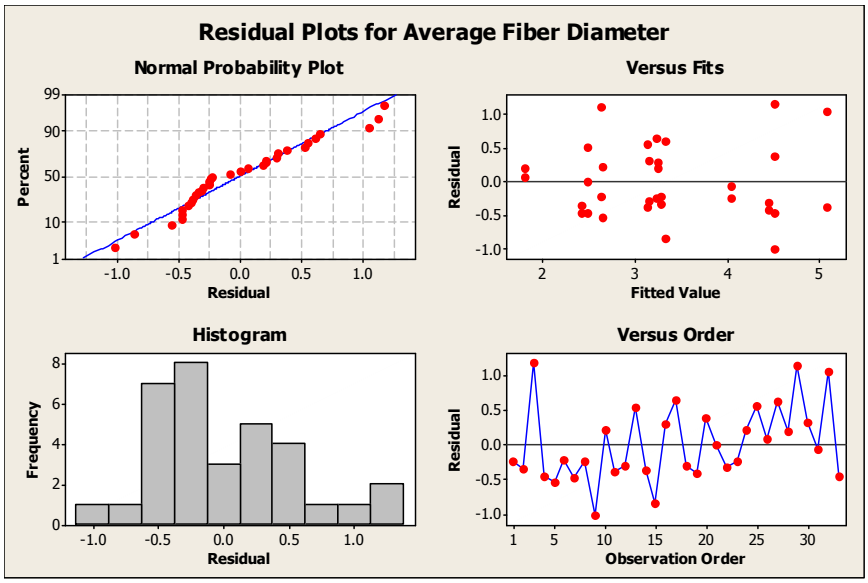
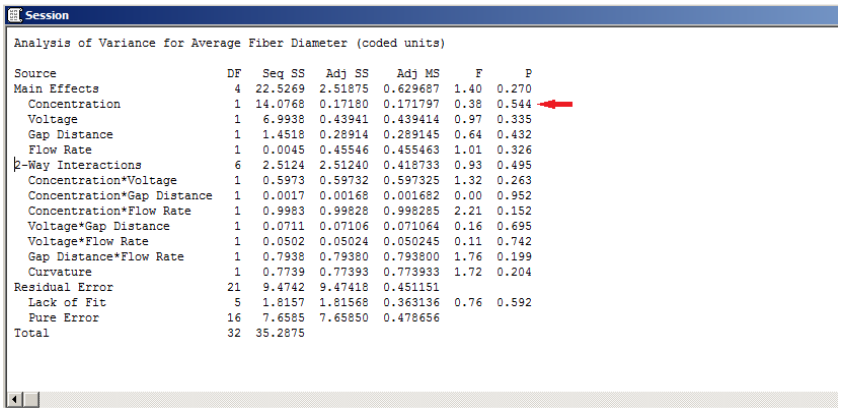


Figure 2: Residual Normal Probability Plot, Versus Fits Plot, Histogram of Residuals, & Run order

2. Theoretical Model

With a 2^4 full factorial, a model will contain a mean term, all 4 main effect terms, all 6 2-factor interaction terms, all 4 3-factor interaction terms, and the 4-factor interaction term. However, initially the assumption that all 3-factor and higher interaction terms are non-existent should be made (it is rare for such high-order interactions to be significant and are difficult to interpret). Minitab reports a p-value for each interaction term, which can be used to determine its significance (Figure 3). If the value is less than .05, the corresponding term would have a significant effect on the fiber diameter. This allows an accumulation of the sum of squares for these terms to be estimated in the error term. The theoretical model to be used will then have 11 unknown constants in which the data is predicted to clarify which are significant main effects and interactions. Also, in the Analysis of Variance, an R^2 value will be calculated. This value must be relatively high because its purpose is to indicate the variability of the prediction of future outcomes based on the current information. If R^2 is low, it indicates that there may be other independent variables

that affect the dependent variable (fiber diameter) besides the factors being investigated (14).



Session

Analysis of Variance for Average Fiber Diameter (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	22.5269	2.51875	0.629687	1.40	0.270
Concentration	1	14.0768	0.17180	0.171797	0.38	0.544
Voltage	1	6.9938	0.43941	0.439414	0.97	0.335
Gap Distance	1	1.4518	0.28914	0.289145	0.64	0.432
Flow Rate	1	0.0045	0.45546	0.455463	1.01	0.326
2-Way Interactions	6	2.5124	2.51240	0.418733	0.93	0.495
Concentration*Voltage	1	0.5973	0.59732	0.597325	1.32	0.263
Concentration*Gap Distance	1	0.0017	0.00168	0.001682	0.00	0.952
Concentration*Flow Rate	1	0.9983	0.99828	0.998285	2.21	0.152
Voltage*Gap Distance	1	0.0711	0.07106	0.071064	0.16	0.695
Voltage*Flow Rate	1	0.0502	0.05024	0.050245	0.11	0.742
Gap Distance*Flow Rate	1	0.7938	0.79380	0.793800	1.76	0.199
Curvature	1	0.7739	0.77393	0.773933	1.72	0.204
Residual Error	21	9.4742	9.47418	0.451151		
Lack of Fit	5	1.8157	1.81568	0.363136	0.76	0.592
Pure Error	16	7.6585	7.65850	0.478656		
Total	32	35.2875				

Figure 3: Minitab's Session Window (p-value for main effects & 2-way interaction)

3. A model can be selected in which only the most important factors are included. The previous step (significance of p-values) is a good indicator of these factors. Minitab's Stepwise Regression tool can help remove unnecessary terms (Appendix C). Minitab will produce Fiber Diameter as a response to the effects that have a greater significance level than .15 (standard value of significance). The value of R2 will assumingly be adjusted to a number closer to 1.0 or 100%, reducing the variability of the predictors.
4. Before conclusions can be made, the model assumptions must be tested using residual graphs and a normality test. In the analysis of the factorial design, residuals were stored in a specified column. A normality test for these residuals should be performed (Appendix C). In a normality test, the null hypothesis is that the residuals are normally distributed therefore a high p-value (>.05) would confirm our assumption. This would allow conclusions to be made in the examined the ANOVA.
5. Use the results to answer the questions in your experimental objective (which factors had the greatest effect on fiber diameter). If desired, an optimization plot can be performed, in which Minitab will calculate parameter values that are predicted to

minimize the resultant fiber diameter (Appendix C). An experimental spin at these settings may be run to test the level of prediction.

Future Work Recommendations

When this experiment has been completed and conclusions are made of the effects that each parameter has on resulting fiber diameter, information from the analysis should be used to determine what setting future spins should be performed at. If certain parameters had little or no effects on fiber diameter, they can be kept as a control, while those parameters that had significant effects can be tested at new levels. Other factors have been known to have effects on fiber diameter include temperature, humidity, and needle size, as previously stated. These factors have not been investigated with the specific electrospinning device at Cal Poly. These factors could be tested using the same methods as this DOE and would help establish the optimal settings to minimize fiber diameter. Another imperative feature of the electrospinning device and process that should be considered is its consistency from trial-to-trial. A consistency study is recommended to be tested at a control setting that is thought to minimize fiber diameter. If there is high variability in the results, a reexamination of the methods and preparation of the electrospinning process must be implemented.

Appendix A

Using Minitab, two-sample t-tests were performed between Edward Siemens's spins to detect a statistical significance between resultant mean fiber diameters. If the p-value for a t-test is below .05, there is enough evidence to prove the two mean fiber diameters are statistically different. No conclusions about compared mean fiber diameters can be made for t-tests that produce p-values greater than .05.

Two-Sample T-Test and CI (Spin 1 vs 2)

```
Sample    N  Mean  StDev  SE Mean
1       261  3.74   1.97     0.12
2       288  3.39   1.47    0.087


Difference = mu (1) - mu (2)
Estimate for difference:  0.345
95% CI for difference:  (0.051, 0.639)
T-Test of difference = 0 (vs not =): T-Value = 2.30  P-Value = 0.022  DF = 477
```

Two-Sample T-Test and CI (Spin 2 vs Spin 3)

```
Sample    N  Mean  StDev  SE Mean
1       288  3.43   2.01     0.12
2       288  3.39   1.47    0.087


Difference = mu (1) - mu (2)
Estimate for difference:  0.033
95% CI for difference:  (-0.256, 0.322)
T-Test of difference = 0 (vs not =): T-Value = 0.22
P-Value = 0.823->(insignificant) DF = 525
```

Two-Sample T-Test and CI (Spin 3 vs 4)

```
Sample    N  Mean  StDev  SE Mean
1       297  2.14   1.42    0.082
2       288  3.43   2.01     0.12


Difference = mu (1) - mu (2)
Estimate for difference:  -1.285
95% CI for difference:  (-1.569, -1.001)
T-Test of difference = 0 (vs not =): T-Value = -8.90  P-Value = 0.000  DF = 514
```

Two-Sample T-Test and CI (Spin 4 vs 7)

```
Sample    N  Mean  StDev  SE Mean
1       296  2.02   1.00    0.058
2       297  2.14   1.42    0.082


Difference = mu (1) - mu (2)
Estimate for difference:  -0.124
95% CI for difference:  (-0.322, 0.074)
T-Test of difference = 0 (vs not =): T-Value = -1.23
P-Value = 0.219-> insignificant DF = 531
```

Two-Sample T-Test and CI (Spin 3 vs 7)

```
Sample    N  Mean  StDev  SE Mean
1       296  2.02   1.00    0.058
2       288  3.43   2.01     0.12


Difference = mu (1) - mu (2)
Estimate for difference:  -1.409
95% CI for difference:  (-1.669, -1.149)
T-Test of difference = 0 (vs not =): T-Value = -10.67  P-Value = 0.000  DF =
     418
```

Two-Sample T-Test and CI (Spin 2 vs 5) -> Increased Sol. Conc.

```
Sample    N  Mean  StDev  SE Mean
1       288  3.39   1.47    0.087
2       288  2.64   1.08    0.064


Difference = mu (1) - mu (2)
Estimate for difference:  0.753
95% CI for difference:  (0.542, 0.964)
T-Test of difference = 0 (vs not =): T-Value = 7.01  P-Value = 0.000  DF = 526
```

Appendix B: Electrospinning Procedure

(From Tiffany Richelle Pena)

1. Calculate the amount of PLGA resin necessary for the desired weight percent polymer solution using the following equation. (Density of chloroform is 1.48 g/ml.)

WPP = m1/ (m1 + m2b)
WPP = Weight percent polymer solution
m1 = mass of polymer (g)
m2 = mass of solvent (ml)
b = density of solvent (g/ml)

2. Put on safety gloves. (*WARNING: Chloroform can have serious side-effects if it comes in contact with skin, eyes or is inhaled or swallowed. Target organs to be effected are kidneys, heart, central nervous system, liver, eyes, reproductive system and skin. Always open chloroform in a hood and wear protective clothing!!*)
3. Remove PLGA (from the freezer and allow it to reach room temperature (5-10 minutes). Doing so prevents condensation when the polymer is exposed to air.
4. Weigh out the calculated amount of PLGA using the Acculab Balance and place the polymer in a 20 ml clear vial. Close the lid immediately.
5. Return unused PLGA to the freezer.
6. Retrieve the chloroform for the hazardous chemical cabinet and place it in the fume hood immediately.
7. Gather the Pipette-Aid, a 10 ml disposable pipette and the vial of weighed PLGA and place in the hood with the chloroform.
8. Pipette the desired volume of chloroform into the vial with PLGA. Immediately cap the vial as well as the chloroform container to prevent evaporation of chloroform since it is highly volatile.
9. Properly label the solution vial with the WPP, date and your initials
10. Wrap vial in aluminum foil to prevent light from entering the solution (chloroform is

highly sensitive to light).
11. Place the vial on the orbital shake table. Set the shake table to approximately 3 revolutions per second. Use tape to ensure that the vial will stay upright while on the shake table. Turn the table on.
12. Allow the solution to mix for 24 hours. After mixing is complete, the solution is usable for up to 48 hours.
13. Remove chloroform container from hood and place back into chemical cabinet.
14. Properly dispose of pipette tip.
15. Clean up work area.

Electrospinning Protocol
WARNING: This electrospinning process requires extremely high voltages! Always wear shoes, gloves, and be mindful of what you are touching. Do not attempt to use the electrospinner unless a qualified user has trained you.

1. Put on safety gloves and protective clothing. (***WARNING****: Chloroform can have serious side-effects if it comes in contact with skin, eyes or is inhaled or swallowed. Target organs to be effected are kidneys, heart, central nervous system, liver, eyes, reproductive system and skin. It is possible for residual chloroform to be present on and around the electrospinner during and after a spin!! Make sure to read the MSDS for all chemicals you are working with and know the necessary emergency procedures.*)
2. The green ground wire located on the backside of the collector can be removed by pulling it straight out. Unplug the green ground wire from the collector.
3. The yellow power wire connects the collector to the DC motor control box. The yellow power wire comes off the DC motor control box by unscrewing the connection head. Unscrew the yellow power wire from the DC motor control box.
4. The collector can now be removed from the containment chamber. Remove the collector from the containment chamber and place it on the counter outside the fume hood.
5. During a spin, stray polymer preferentially builds up on exposed metal, wires and the motor casing. Cleaning before each spin is necessary to remove any residual polymer or dust from the collector that may potentially interfere with the next spin process. Clean the collector using IPA and paper towels.
Ensure all residues from both the front and back of the collector including the wires are removed.
6. During a spin, stray polymer can attach to any wall of the containment chamber and even form webs of polymer between walls. If necessary, clean the inside of the containment chamber with IPA.
7. Replace the collector back inside the containment chamber and reconnect the green ground and yellow power wires.
8. Prepare the mandrel for spinning. Clean the mandrel with IPA to remove any dust, residual polymer from a previous spin, or metal particles. If the mandrel surface is scratched, fine grit sand paper can be used to re-smooth the surface of the mandrel. If sanding is necessary, clean the mandrel with IPA when finished.
9. Attach the mandrel to the collector. When inserting the mandrel, rotate the turn knob until most of the metal chuck is covered, you will feel some resistance. If you go too far the turn knob will spring back.
10. There are three power cords to the right of the fume hood; one for the external power supply, one for the main power and one for the DC motor control box. Plug in all the equipment.
11. Using a multimeter, check the resistance between the ground connection and the

mandrel. Verify that there is some conductivity. Record your measurements.
12. In the fume hood, prepare a 10 ml syringe with an 18GA fill needle.
13. Remove aluminum foil from the PLGA solution vial for better visibility when working with the solution.
14. Solutions may be highly viscous and filling the syringe may take time and require some strength. Make sure to not release pressure on the plunger when drawing solution into the syringe. Acquire just over 3 ml of the polymer solution into the prepared 10 ml syringe
15. Once the solution has been acquired in the syringe, replace the fill needle with an 18GA Blunt needle.
16. Push the plunger back into the syringe until most of the air is removed and the solution is just in the needle. ***WARNING****: If you push too hard too fast the polymer melt may squirt out. If this happens you will need to attach a new needle.*
17. Place the filled 10 ml syringe into the syringe pump. The needle should go through the needle tip hole in the containment chamber wall.
18. Re-position the collector in the containment chamber so that the mandrel and the needle tip are 10 inches apart and perpendicular to each other. *NOTE: The side of the collector with green ground wire connection should face away from the needle.*
19. Hang the exposed metal of the red high voltage wire on the needle tip inside the containment chamber. You can secure the wire on the needle by taping the wire to the containment chamber wall. ***WARNING****: If the wire falls off the needle during the spin, the external 10 V power source used to regulate high voltage output will burn out. Be sure to hang the wire on the needle securely!*
20. The power switch for the syringe pump is located on the back of the pump. Turn the syringe pump on.
21. Enter the desired flow rate and solution volume. The solution volume will determine when the pump will stop. Make sure the screen remains on volume. *NOTE: Syringe pump instructions are located in a cabinet close to the fume hood if you need further instructions*.
22. Turn on the "Rotate" and "Slide" functions of the collector at the DC motor control box. Ensure the collector is now oscillating back and forth and the mandrel is rotating. If the mandrel is not rotating, you can tap it gently to get it started.
23. Place the front wall onto the containment chamber. The bottom right corner of the front wall is cut for wires to run through. Secure the wall in place with tape so it does not come off.
24. Turn on the external power source and set it to the desired voltage. Turn the external power source off.

Read Steps 25-40 BEFORE beginning the electrospinning process. The following steps for turning ON and OFF the electrospinning system must be followed in the exact order listed.

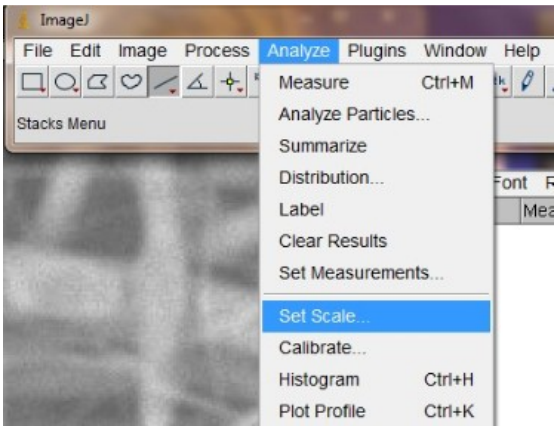
25. Press the "Run" button on the syringe pump. The volume count will begin on the screen and an arrow will flash meaning the solution is now being pushed through the needle.
26. When a droplet forms on the tip of the needle, the process is ready to begin.
27. Turn on the "Main Power" (left switch). Power is on if the light on the AC/DC power converter turns green.
28. Turn on the external source.
29. Prepare to turn on the High Voltage (right switch). Look at the droplet of polymer on the end of the syringe and turn High Voltage on. The droplet should disappear.
30. Monitor the process for the entire spin. The mandrel should slowly start to become coated with the polymer.
31. When the entire polymer has been spun, the process should be shut down in the following order.

a. Press the RUN/STOP button on the syringe pump.
b. Turn the HV switch to OFF
c. Turn the Main Power OFF
d. Turn the ROTATE and SLIDE functions on the DC Motor Control box OFF.
32. In order to allow the solvent to fully evaporate, wait a few minutes before removing the mandrel from the containment chamber.
33. Remove the front containment chamber wall.
34. Remove the mandrel with PLGA scaffold.
35. Touch the red high voltage wire to the green ground wire to remove any residual charge.
36. Remove syringe from syringe pump and dispose in sharps container.
37. Unplug all equipment.
38. Properly dispose of all waste and clean up your workstation.
39. Transfer the mandrel with the PLGA scaffold to the desiccators for further drying of the scaffold. Allow the scaffold to remain on the desiccators for 24 hours.
40. Remove the scaffold from the mandrel using gauze and carefully twist the scaffold off.

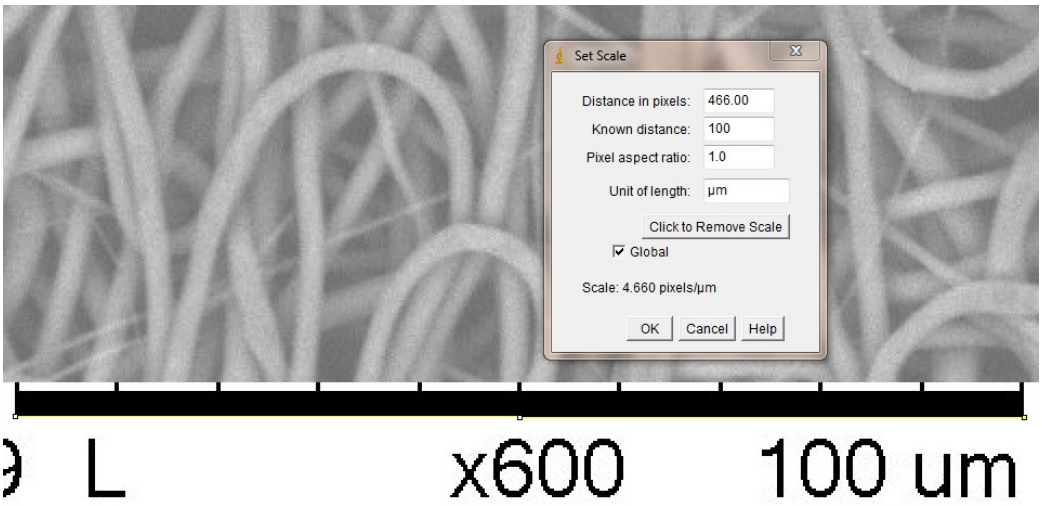
SEM Analysis (From Edward Siemens Protocol)

ImageJ protocol
ImageJ 1.44i was used to measure fiber diameter. The following protocol was used to measure fiber diameter size:

Firstly, the scale must be correlated to the scale of the image as taken by the SEM. After opening the first file in the series with ImageJ, we select the *straight* tool used to create segmented lines by freehand. The image is then zoomed in to a considerable size so that an accurate measurement could be made. After precisely drawing a line the entire distance of the scale bar, we open the “Analyze” tab and select “Set Scale…” from the drop down menu.

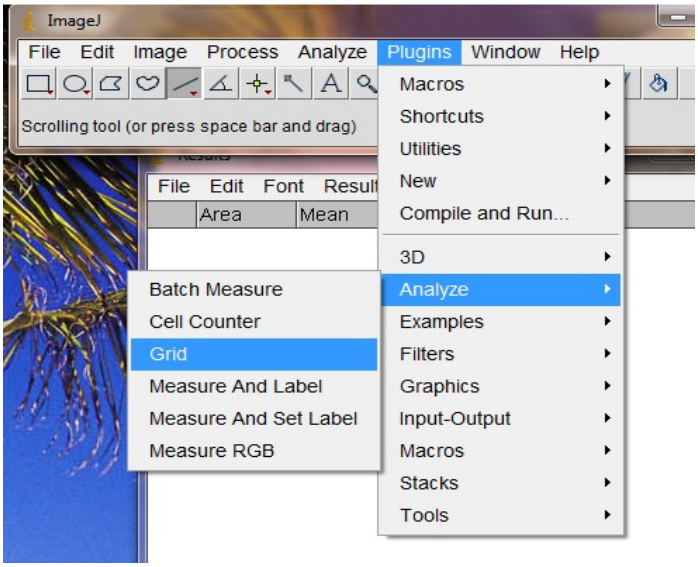


The window opened tells us how many pixels our line segment is that we drew. We then enter the known distance (in this case 100) and the unit of length (in this case µm or microns). Since all images in each series were taken at the same magnification (x600) we can check the “Global” box which will allow us to move to the next image without losing our scale.

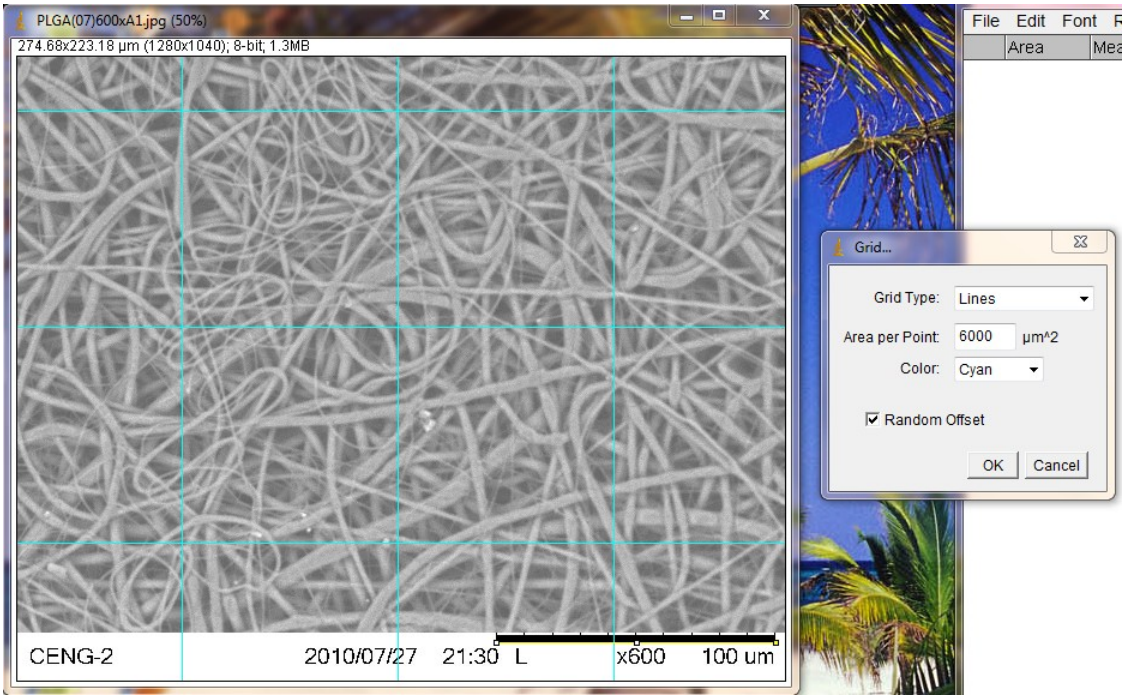


To remove any bias towards selecting desirable fibers for more favorable results we need to set up a random selection of fibers to measure. To do this, a grid was set up which could randomly be placed over the image to select nine points on the image to be measured. Nine points on each image would give nine fibers times eight images on four sections of a scaffold which would be two hundred eighty-eight measurements; this gives us a statistically relevant sample size.

To create this grid we open the "Plugins" tab, hover over "Analyze," and select "Grid."

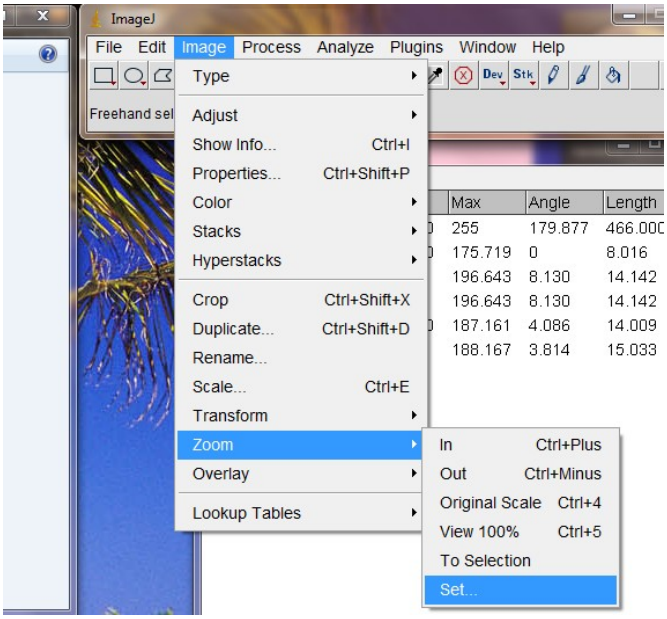


As seen in the following image, a crosshatch pattern is laid out over the image. The larger the number in the "Area per Point" is, the larger the squares of the crosshatch will be. The value of 6000 gave nine easily distinguishable points which were spread out over the image and so that is the value we used here.

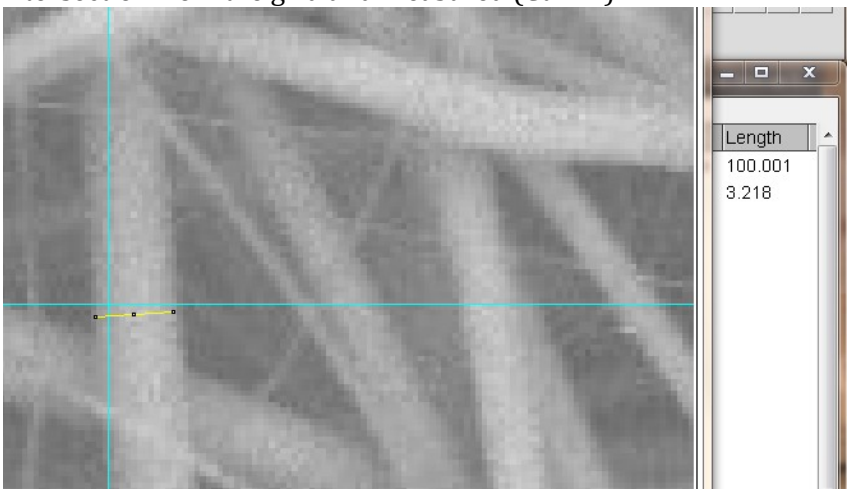


We are then ready to take measurements. To make sure our scale is correct before continuing, we draw a line segment across the measure bar on the image and hit Ctrl-M. This should cause a window to pop up with the measurement. If it does not, click on the "Analyze" tab and select "Measure." Our measurement should read approximately what we set our scale to. In this case we have 100.001µm which is very close to our known distance of 100µm.

At this scale, one pixel could mean the difference between 3µm and 4µm and thus very inaccurate. To make precise measurements we will need to zoom in quite a bit. Select the "Image" tab, hover over "Zoom," and select "Set…."

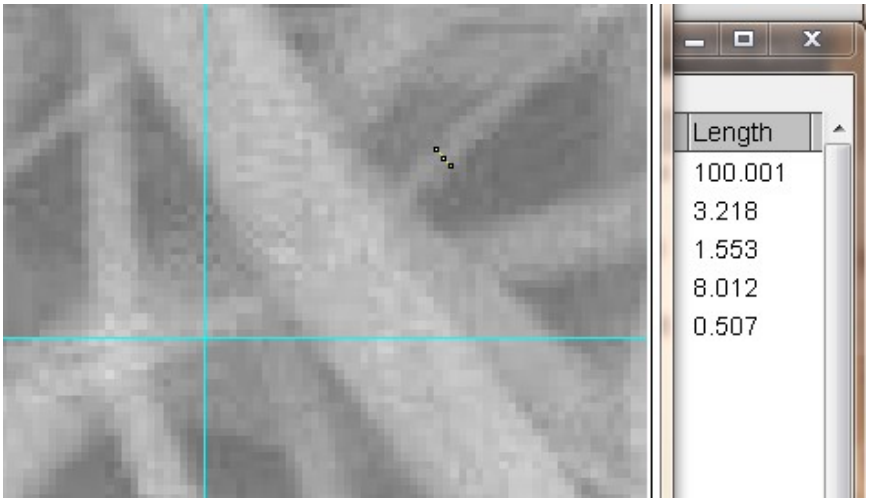


We can then set how much we want the image to be zoomed in. The value of 450-550 was most often used, depending upon how small the fiber sizes were for that particular spin. The following images were done at 450x. A line segment is drawn across the fiber at the point of intersection from the grid and measured (Ctrl-M).



Tip to move the image around to the next intersection, the SpaceBar can be held down which will allow free movement of the image with the mouse.

At times an intersection could point to a fiber which could not be accurately measured at that point due to other fibers interfering in the visibility of the width of the selected fiber. In these cases the particular fiber can be traced to a point where visibility is better and measured there. As shown in the following image, the smaller diameter fibers can be quite hard to measure accurately and so further zooming would be necessary as one pixel may change the measurement drastically.



After all nine points on the image were measured the next image was opened with ImageJ (Ctrl+Shift-O). When all thirty-two images were completed, the data was saved as a txt file which was then inputted into excel.

Appendix C: Creating & Analyzing a DOE Using Minitab

To create a Factorial Design (Figure 4):

Open Minitab 16.1.1> Stat > DOE > Create Factorial Design

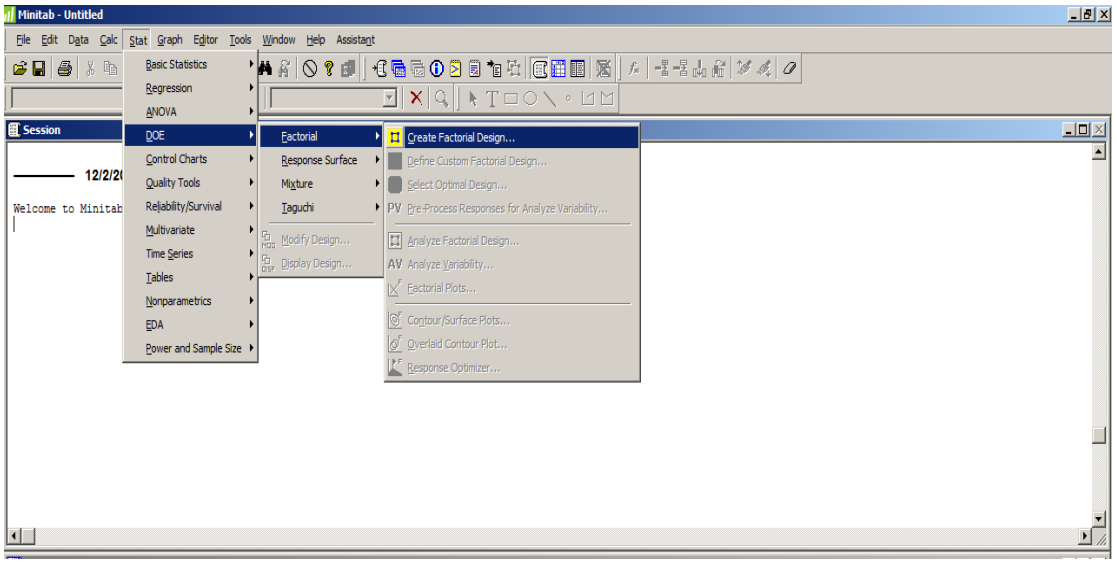


Figure 4: Selecting Factorial Design

Once selected, individual can select number of factors, levels, center points, and replications. Factors can be labeled and factor level quantities can be set. Minitab then generates the design and automatically randomizes the run order to eliminate procedural bias.

Worksheet 2 ***

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
	StdOrder	RunOrder	CenterPt	Blocks	Concentration	Voltage	Gap Distance	Flow Rate					
1	2	1	1	1	15.0	24	7.0	4.5					
2	8	2	1	1	15.0	30	10.0	4.5					
3	5	3	1	1	10.0	24	10.0	4.5					
4	17	4	1	1	10.0	24	7.0	4.5					
5	28	5	1	1	15.0	30	7.0	6.5					
6	20	6	1	1	15.0	30	7.0	4.5					
7	24	7	1	1	15.0	30	10.0	4.5					
8	27	8	1	1	10.0	30	7.0	6.5					
9	21	9	1	1	10.0	24	10.0	4.5					
10	6	10	1	1	15.0	24	10.0	4.5					
11	25	11	1	1	10.0	24	7.0	6.5					
12	26	12	1	1	15.0	24	7.0	6.5					
13	14	13	1	1	15.0	24	10.0	6.5					
14	23	14	1	1	10.0	30	10.0	4.5					
15	3	15	1	1	10.0	30	7.0	4.5					
16	22	16	1	1	15.0	24	10.0	4.5					

Figure 5: Minitab output for DOE

Once experiment is complete, corresponding results can be input in C9 (highlighted region in Figure 5). Minitab can then be used to analyze the factorial design with the following actions:

Stats>DOE>Analyze Factorial Design → Enter C9 in 'Responses'
->Terms: to be analyzed can be selected as displayed in Figure 6 (select up to 2nd order)
-> Storage.. -> Check "Residuals"

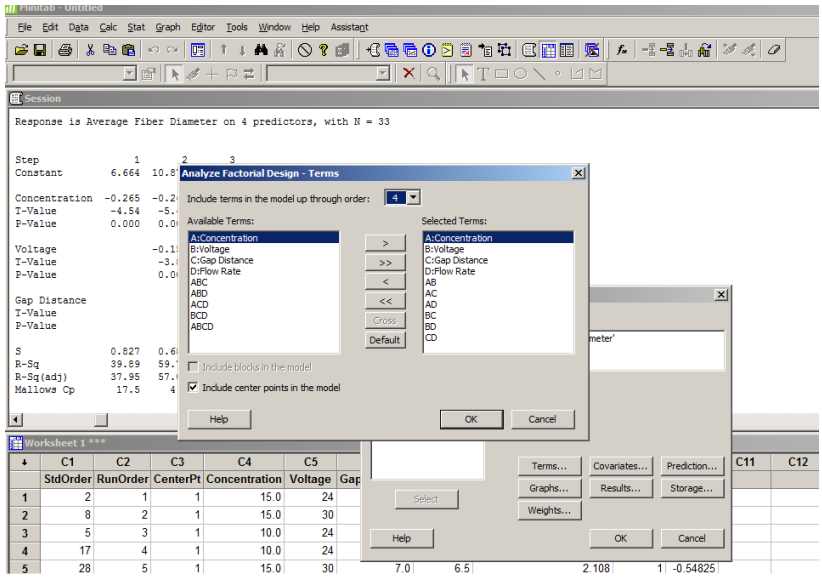
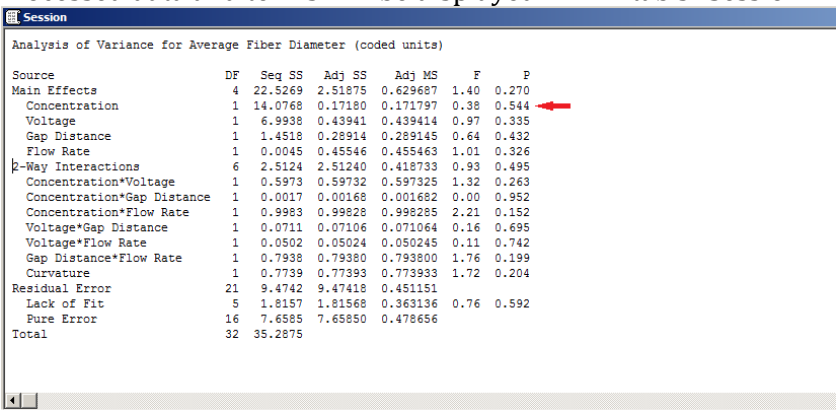


Figure 6: Selecting Terms to be Analyzed

Processed data and terms will be displayed in Minitab's "Session" window (Figure 7).



Session

Analysis of Variance for Average Fiber Diameter (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	22.5269	2.51875	0.629687	1.40	0.270
Concentration	1	14.0768	0.17180	0.171797	0.38	0.544
Voltage	1	6.9938	0.43941	0.439414	0.97	0.335
Gap Distance	1	1.4518	0.28914	0.289145	0.64	0.432
Flow Rate	1	0.0045	0.45546	0.455463	1.01	0.326
2-Way Interactions	6	2.5124	2.51240	0.418733	0.93	0.495
Concentration*Voltage	1	0.5973	0.59732	0.597325	1.32	0.263
Concentration*Gap Distance	1	0.0017	0.00168	0.001682	0.00	0.952
Concentration*Flow Rate	1	0.9983	0.99828	0.998285	2.21	0.152
Voltage*Gap Distance	1	0.0711	0.07106	0.071064	0.16	0.695
Voltage*Flow Rate	1	0.0502	0.05024	0.050245	0.11	0.742
Gap Distance*Flow Rate	1	0.7938	0.79380	0.793800	1.76	0.199
Curvature	1	0.7739	0.77393	0.773933	1.72	0.204
Residual Error	21	9.4742	9.47418	0.451151		
Lack of Fit	5	1.8157	1.81568	0.363136	0.76	0.592
Pure Error	16	7.6585	7.65850	0.478656		
Total	32	35.2875				

Figure 7: Minitab's Output of DOE Analysis in Session Window

To create interaction plots (figure 8):

Stats>ANOVA>Main Effects Plot -> select all factors

Stats>ANOVA>Interaction Effects Plot -> select all factors

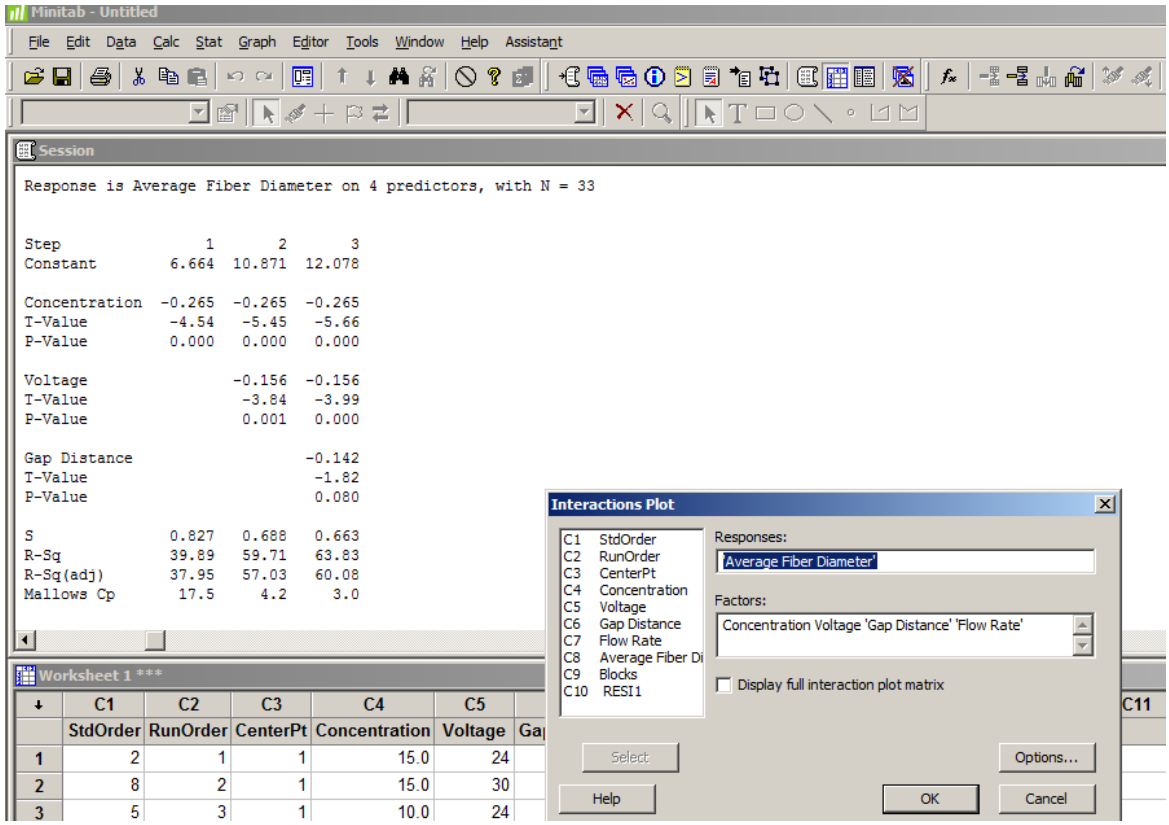


Figure 8: To create Main effects & Interaction Effects Plots

To perform a stepwise Regression on Minitab (Figure 9):

Stats>Regression>Stepwise Regression -> Factors as Predictors; Fiber Diameter as Response

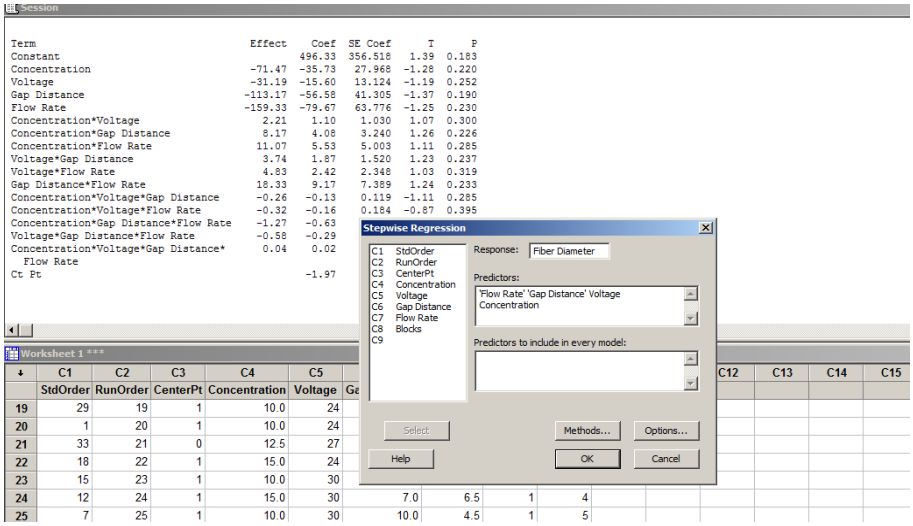


Figure 9: Input for Stepwise Regression

To perform a Normality test for Residuals (Figure 10):

Graph>Probability Plot – Single – Input RESI 1

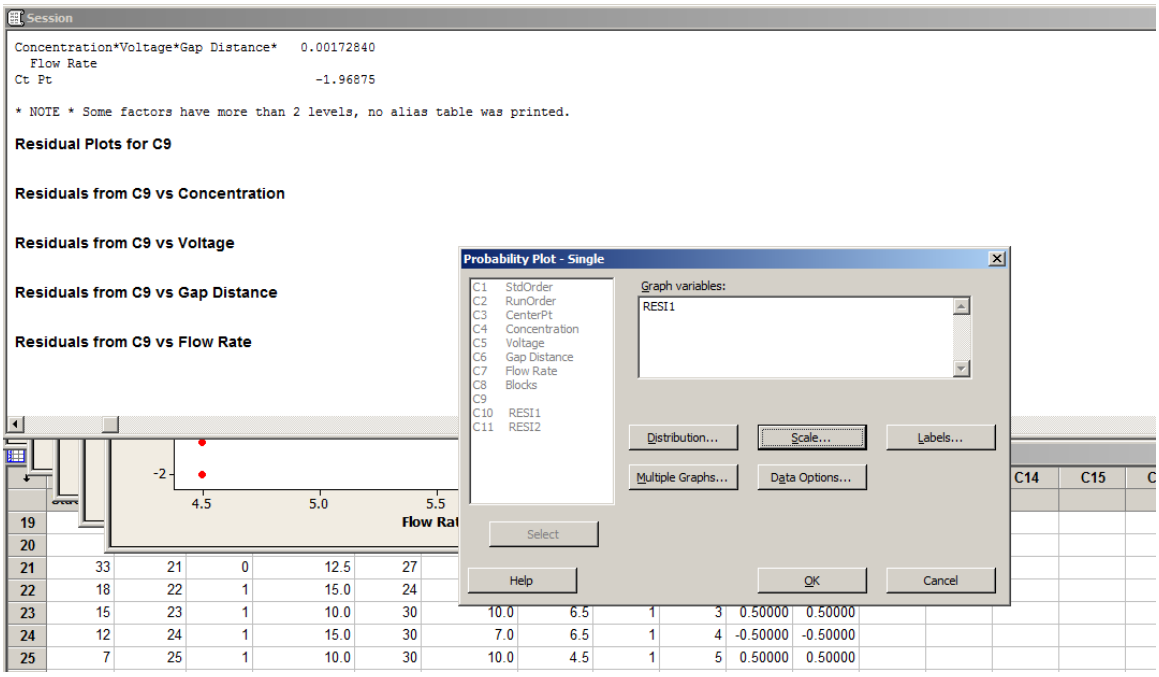


Figure 10: To Perform Normality Test of Residuals

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