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| 14. ABSTRACT<br>A need exists for a portable sterilization system that can quickly sterilize skin for possible use with medical instruments and skin catheters. To address this challenge, MicroStructure Technologies (MicroST) is developing an atmospheric pressure nonthermal plasmas for rapid deactivation of cells and bacterial spores on surfaces. For example, the lethal Ames strain of <i>Bacillus anthracis</i> has been deactivated after a minute of ionized gas exposure. The objective of this DARPA seedling project is to demonstrate the sterilization efficiency of novel plasma blanket designs on: <ul style="list-style-type: none"> <li>Inoculant bacteria such as <i>Staphylococcus</i> and possibly <i>Leishmaniasis</i>.</li> <li>Bacterial spores require longer exposures to ionized gases to effect sterilization</li> <li>The inoculant shall be placed on the top and/or bottom of artificial skin to reveal surface and subcutaneous sterilization capability of the ionized gas treatment.</li> </ul> The plasma blanket succeeded in demonstrating close to a log 3 reduction in bacterial cells on skin samples. |             |                                |                               |                                                          |                                                                |
| 15. SUBJECT TERMS<br>Sterilization, ionized gas discharge, plasma, bacterial spores, vegetative cells                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |             |                                |                               |                                                          |                                                                |
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May 6, 2007

**Title of Project:** DARPA – Sterilization of Medical Instruments  
**Contract:** # FA9550-06-C-0054  
**Principal Investigator:** Joseph Birmingham  
**Report:** FINAL Report

## **1. Executive Summary**

### ***Identification & Significance of the Opportunity***

A need exists for a portable sterilization system that can quickly sterilize skin as well as medical instruments and skin catheters. To address this challenge, MicroStructure Technologies (MicroST) is proposing a compact, low maintenance, and high efficiency plasma blanket to satisfy the following needs:

1. To demonstrate that plasma can kill pathogens efficaciously on biological surfaces without damage to the biological surface.
2. To understand the mechanism which kills the spores (the role of electric fields and electrical properties) because this influences device design, expected performance, and a safety and use profile for any potential device.
3. To identify the penetration depth efficiency. Is it just primarily a surface phenomenon or is plasma efficacious at treating below an exposed biological surface? Could several layers of skin be barrier to sterilization?

It has taken more time and resources than expected to establish the first (and primary) objective. Setting up the lab and procedures to demonstrate at minimum a log 3 reduction in bacteria through culturing, inoculating and plasma-treating artificial skin, extracting, diluting, plating and counting colonies has taken careful planning and effort to achieve in a few months for modest costs.

The DARPA program manager emphasized quantitative statistics and rigorous error analysis over proof-of-principle experiments. We showed step-by-step error analysis of our source-concentration determination, which has been used to validate our dilution and plating protocols. We have evaluated several of the experimental steps, and have improved source delivery methods, which also has boosted efficiency of extraction of bacteria from the artificial skin. These changes necessitated changing plasma treatment parameters, so it has been necessary to re-investigate plasma treatment times to achieve the requested levels of bacteria reduction. The DARPA PM made it clear that complete elimination of bacteria, i.e. no growth on cultured plates, was not desired at this time. What he wanted to see first was a level of reduction that left a small (but countable) number of viable bacteria on the treated plates. To achieve this and show log 3 reduction it is necessary to dilute the exposure controls by three orders of magnitude and have ~E2 remaining to grow on the exposure-control plates, while finding plasma treatment conditions that plate (without dilution) to E1. We have succeeded in boosting source concentration as well as extraction efficiency to achieve this. We have found a plasma treatment condition that gives the desired E1 when plated. What remains is to test repeatability with a statistically significant number of runs.

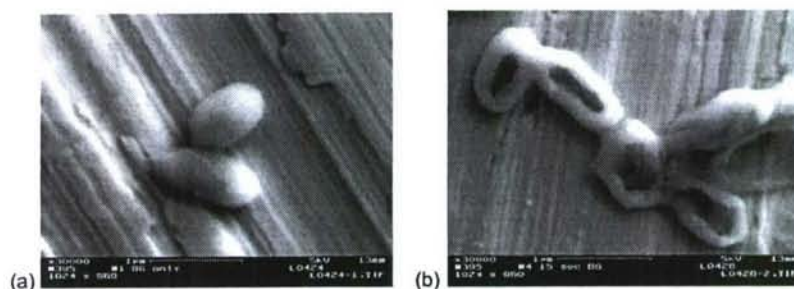
## Proposed Approach: Demonstrate Ionized Gas Sterilization of Artificial Skin

The objective of this DARPA seedling project is to demonstrate the sterilization efficiency of novel plasma blanket designs on:

- Inoculant bacteria such as *Staphylococcus* and possibly *Leishmaniasis*.
- The inoculant shall be placed on the top and/or bottom of artificial skin to reveal surface and subcutaneous sterilization capability of the ionized gas treatment.

The goal of this project is to demonstrate a flexible plasma reactor to wrap around body parts that are infected and promote sterilization of skin.

The miniaturized plasma lysis approach uses a low temperature, atmospheric pressure air purification system using only a few watts of power (Birmingham and Hammerstrom, 2000a, 2000b). We have demonstrated the lysing capability of plasma impinging on a surface contaminated with biological materials. Figure 1 shows *Bacillus subtilis* (Bg) bacterial spores that have been lysed with 15 seconds of ionized gas exposure. The biological membranes are ruptured after exposure to the plasma environment.



**Figure 1.** (left) SEM micrograph of Bg Spores Prior to Treatment by the MicroST Plasma Lysis System and (right) after 15 Seconds of Treatment.

## SUMMARY OF PHASE I ACCOMPLISHMENTS

Several measurements of plasma sterilization of *Staphylococcus epidermis* (Staph):

### A. The sample extraction efficiencies:

MicroST concentrated efforts on culturing cells for reduced error rates to achieve precise quantitative results. The mixing was needed to make accurate dilutions with NaCl in 0.85% concentration in sterile water for final concentrations. The growth conditions for the *Staphylococcus epidermis* (Staph) were optimized using a shaker with broth of Staph cells included reduced clumping of the Staph for better uniformity of dilutions and plates. Extraction experiments illustrated the importance of using test skin to more accurately determine the removal of bacteria from the skin surface is 11%. The plasma sterilization efficiency was established at 99.9% currently.

B. The establishment of a correlation between optical measurements and plating was attempted without success. The ability to correlate the stain-assisted concentrations of cells with optical microscopy has been determined to be suspect. It was found that the stain suffered photon bleaching that perturbed the measurements.



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## 2. Experimental Approach

The study of ionized gas interactions with artificial skin require completion of several tasks designed to address the project objectives. As shown in Table 1, the experiments are intended to determine the extent to which non-thermal atmospheric pressure plasma may be used to sterilize skin from infectious agents. Another key question that needs to be addressed is whether the effect extends below the skin surface. The following are suggested as an initial set of experiments that should provide sufficient information to that end. Additional experiments may be proposed given sufficient time and/or resources to complete them (e.g., efficacy against other pathogenic agents). Experiments may be performed with artificial skin (e.g., Integra), cadaver skin, or animal skin of carefully controlled thickness.

**Table 1.** Experimental Parameters

| Experimental Controls         |                                                                                  |                                  |                                                                    | Measurements for each control                                           |                                                                                                 |
|-------------------------------|----------------------------------------------------------------------------------|----------------------------------|--------------------------------------------------------------------|-------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Tissue Depth                  | Gas Composition                                                                  | Plasma / E-Field                 | Exposure Time                                                      | Electrical Properties                                                   | Biology Impact                                                                                  |
| -0mm<br>-1mm<br>-3mm (or 5mm) | -Normal Atmosphere<br>-Pure Nitrogen<br>-N <sub>2</sub> / O <sub>2</sub> Mixture | -Plasma<br>-Pulsed E-field alone | -Several seconds<br>-If no effect, a longer exposure (few minutes) | -Device Capacitance<br>-Skin / Device Capacitance<br>-Power dissipation | -Fraction of Agent Survived<br>-Any additional measurements necessary to confirm bacteria death |

### Task 1. Design and Fabricate Plasma Blanket:

#### DESIGN AND FABRICATION OF THE PLASMA LYSER

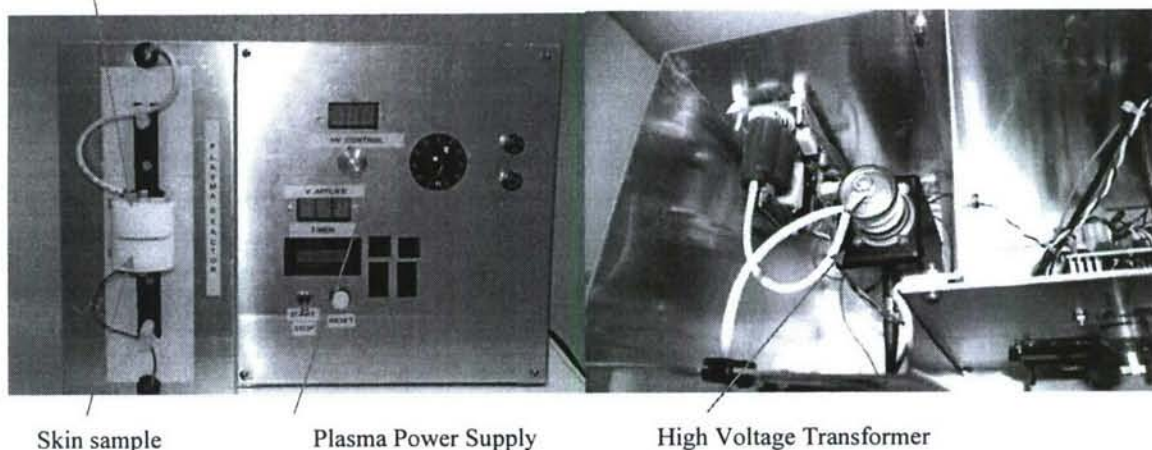
A preliminary prototype plasma lyser has been completed as shown in Figure 2. During our literature search, MicroStructure Technologies has determined that the US Patent (# 6,010,554 January 4, 2000 "Apparatus and Method for Lysing Bacterial Spores to Facilitate Their Identification" Birmingham, et.al.) is the basis of more development work.



**Figure 2.** MicroST Plasma Lyser prototype product used for biomaterial extraction and decontamination experiments.

The dielectric barrier (using quartz components) discharge was substituted for the plasma blanket due to the better experimental control needed to achieve the log 3 reduction (see Figure 3). A plasma test stand has been fabricated and is being used to perform exposure tests of *Staphylococcus* cells on artificially grown skin. The test stand has been designed to provide precise location and repeatable spacing of the electrodes for generating reproducible plasma exposure intensity and period. Considerations such as rapid replacement of electrode dielectric barriers for each exposure minimize concerns with test sample contamination. Easily replaceable high voltage power supplies as well as primary voltage adjustment permit a range of voltage conditions to be explored. Secondary current adjustment facilitates sample exposure to plasmas of different intensities. Initially, plasma conditions have been optimized subjectively using indicators such as uniformity (i.e. minimizing micro-arcing), plasma stability without catastrophic arcing, and maximum power and exposure time without charring the skin test sample. Acceptable progress has been realized in dramatically reducing viability of the skin inoculate (i.e. *Staphylococcus* cells) without obvious destruction of the skin sample.

Plasma Barrier Discharge Reactor



**Figure 3.** Photographs of the MicroLyser Experimental Apparatus used for biomaterial treatment experiments.

The range of plasma conditions is inherently limited by the requirement to couple power into the system to initiate and maintain the electrical discharge. While the system has a fixed electrode configuration, spacing and test sample characteristics can impact the RLC (resistance-inductance-capacitance) tank circuit. The measurement of power dissipated by the load (i.e. test sample) and changes in the resonant frequency of the circuit indicate the level of exposure as well as changes (i.e. electrical properties) to the test sample. In the near future, further refinements to the test stand will permit measurements of both dissipated power and circuit frequency. Several challenges are being engaged to implement these electrical diagnostics. First, the design and fabrication of a new high voltage probe is underway. The initial probe was a typical resistive voltage divider design. It has been determined that the frequency response of this probe is unacceptable. The new design utilizes an inverted resistive-capacitive divider design with a frequency response of hundreds of kilohertz. In addition, a high frequency power measuring circuit is being investigated as an alternative to independent, secondary voltage and current measurements.

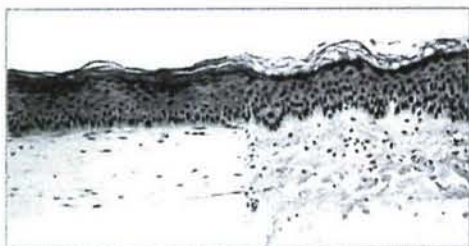
The objective of the plasma parametric work in conjunction with the Staphylococcus cell treatment experiments is to determine the nominal electrical conditions for affective reduction of cell viability. A significant goal of this project is to achieve at least  $10^3$  reduction in inoculate colony forming units (CFU) using plasma conditions which are not hazardous to a human. Consequently, the assessment of feasibility of an economical, flexible plasma bandage which can be applied to even seeping wounds and retain its effectiveness has begun. Another significant goal of this project is to achieve at least  $10^6$  reduction in inoculate CFU using plasma conditions where the potential health hazards can be contained. A second desired outcome of this project is the assessment of feasibility of an economical, flexible plasma blanket for sterilizing medical instruments and surfaces. To this end, an apparatus for customizing electrode material and spacing during lamination has been designed, fabricated, and utilized. Several coplanar electrode configurations have been fabricated and tested. The latest configuration has produced areas of limited plasma activity on the dielectric surfaces between the electrodes. Work is continuing to achieve a configuration of electrode spacing, dielectric materials, and lamination quality that produces a more uniform surface plasma.

The volume resistivity of the gas and dielectrics can be determined by measurements of dissipated power and resonant frequency. The values can be compared with published numbers. Inserting the sample into the test system (i.e. circuit) should change the reactance and thereby the capacitance of the circuit. Both the deposited power and resonant frequency should be affected by this. Comparing the amount of power dissipated in each component of the plasma cell will yield the power dissipated in the skin sample and associated inoculate.

**Task 2. Sterilize Contaminated Artificial Skin: Determine Inoculant Survival on Surface and Subcutaneous locations after Different Amounts of Ionized Gas Exposure, Electric Field Conditions, and Reactor Gas Conditions**

#### SYNTHETIC SKIN PRODUCTS

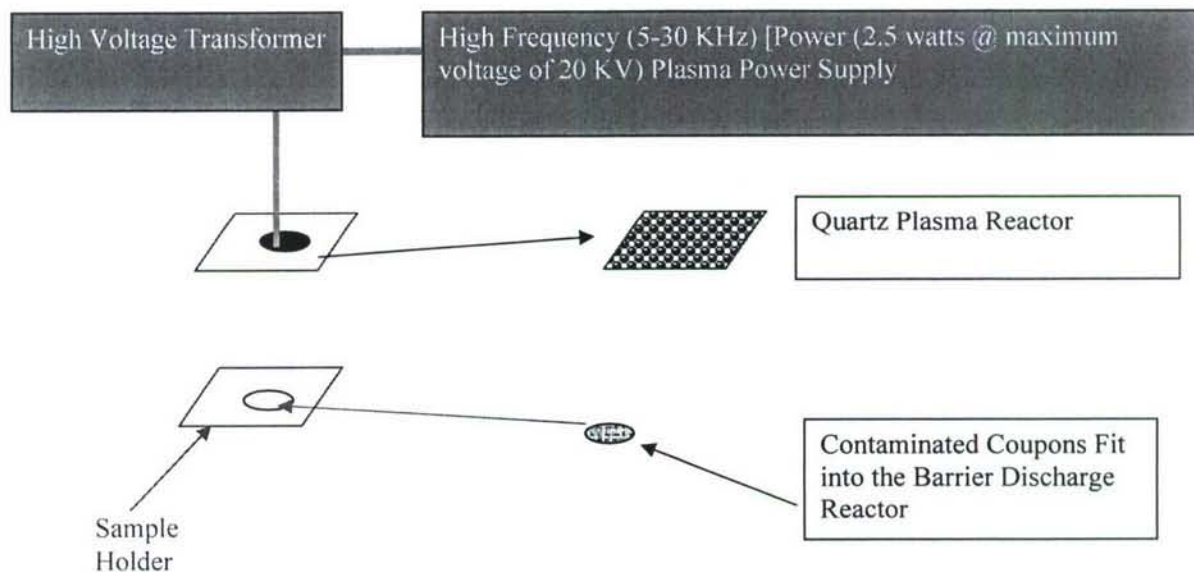
MicroST considered using Integra, a dermal regeneration product. It was decided the structure of Integra was not close enough to human skin to test for any plasma effects. Apligraf, and its research equivalent "Testskin" has a structure very closely resembling human skin making it a good analogue for plasma testing. The Testskin is a living skin equivalent composed of fibroblasts and keratinocytes cultured from human skin. The Testskin has an underlying dermal layer, with partly woven bovine collagen, on which human skin cells are grown. After the 20 days to manufacture Testskin, the product is a ~300-micron thick dermis and an overlying epidermis ~100-250 microns thick. The epidermis even mimics human skin in that the underlayers are living and the upper layers have hardened (cornified) into a protective stratum corneum surface. This structural similarity suggests that the effects of plasma treatment on different skin layers can be assessed. Histologic procedures exist for cross-sectioning and staining skin to examine the different layers. As found in Figure 4, an image of Testskin (left) is next to human skin (right), illustrating the distinct similarity of layers in the dermis and epidermis.



**Figure 4.** Cross-section of Synthetic TestSkin used in these tests- Testskin (left) next to human skin (right), illustrating the distinct similarity of the layers in the dermis and epidermis.

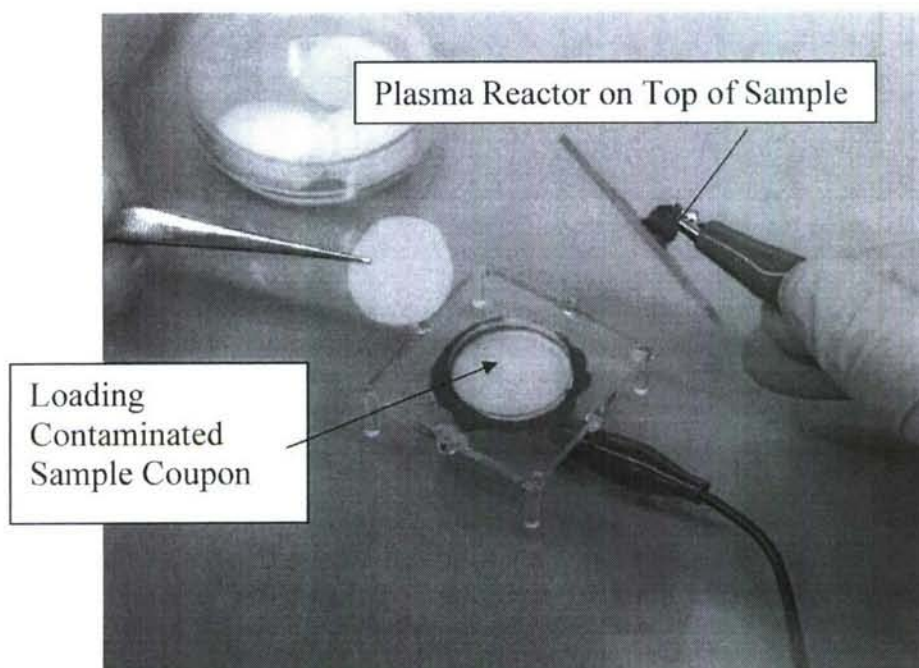
### DESCRIPTION OF SAMPLE PLACEMENT WITHIN PLASMA REACTOR

The flat plate plasma reactor is a dielectric barrier discharge device with embedded metal electrodes as shown in Figure 5. The bottom piece of the plasma reactor is dielectric plate about a quartz cover slip about 1/16-inch thick with a center-to-center electrode spacing that is similar. The contaminated coupon fits into the reactor. A contaminated sample is placed between dielectric barriers of the reactor. The sample coupon thickness is kept constant.



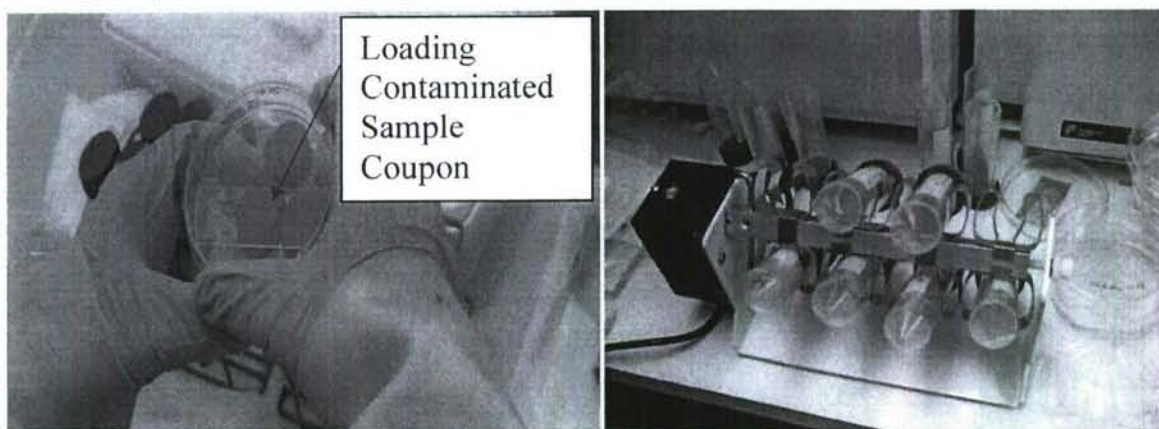
**Figure 5.** Experimental Quartz Plasma Reactor and Plasma Power Supply

Each coupon is contaminated with a 20-microliter drop of biomaterials such as bacterial spores. For example, the 20-microliter drop of  $1.2 \times 10^9$  of *Bacillus* spores or *Staphylococcus* is placed on the center of the sample coupon. The contaminated sample is placed under a plasma reactor for various times of ionized gas exposure as shown in Figure 6.



**Figure 6.** Photograph of Plasma Blanket Reactor loading Contaminated Coupon.

Each coupon is contaminated with a 20-microliter drop of biomaterials such as bacterial spores. For example, the 20-microliter drop of  $1.2 \times 10^9$  of *Bacillus* spores or *Staphylococcus* is placed on the center of the sample coupon. The contaminated sample is placed under a plasma reactor for various times of ionized gas exposure as shown in Figure 7.



**Figure 7.** (Left) Photograph of loading Contaminated Skin Coupon and (Right) Staph Broth in Mixer.

#### DETERMINE EFFICIENCY OF INOCULANT SURVIVAL: EXPERIMENTAL PROCEDURE

To demonstrate sterilization of the plasma blanket unit, MicroST will treat artificial skin surfaces contaminated with biological inoculants such as *Staphylococcus* and possibly *Leishmaniasis* bacteria. The sterilization efficiency (SE) is determined by equation 1:

$$\text{Sterilization Efficiency} = 100 \left( 1 - \frac{[\text{Surviving CFU}]}{[\text{Extractable CFU}]} \right) \quad (1)$$

where the number of surviving colony forming units (CFU) is determined by plating a sample extraction from the plasma-treated skin coupon. Similarly, the number of extractable CFU is determined using control coupons of known bacteria count.

A typical example of skin sterilization may be the deposition of bacteria (i.e. *Staphylococcus* or possibly *Leishmaniasis*) on the surface, followed by destruction upon contact with the ionized gas. Therefore, an artificial skin such as a bilayered skin equivalent (Apligraf or Graftskin) with living epidermis and dermis could be used. One of these artificial skin coupons will serve to determine the sterilization capabilities of the plasma blanket unit. The sterilization test will be conducted on three sets of items. Before contamination, three control test coupons from each test item type will be analyzed to determine the pretreatment bacteria concentration. For determination of the extraction efficiency, three coupons will be sacrificed by placing a known concentration of bacterial compounds deposited by syringe on the artificial skin. Each coupon is swabbed to provide samples for incubation and eventual enumeration (or colony counting). Three more test items will then be treated with the plasma blanket unit for the same amount of time, with the device operating at optimized conditions to sterilize the test items. Control and test items will be swabbed and analyzed to determine the quantity of simulant remaining on each artificial skin item. With this data, an assessment of the plasma blanket unit's ability to sterilize the test items will be made.

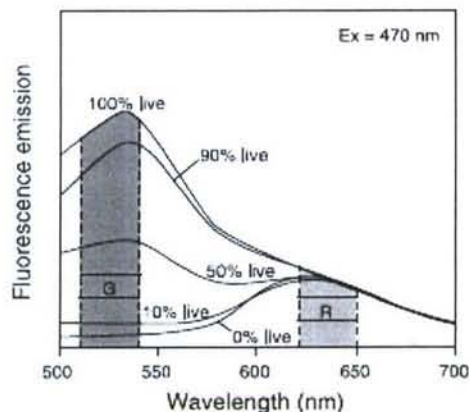
#### **LIVE VERSUS DEAD BACTERIAL TESTING OPTICAL : INITIAL APPROACH DISCARDED**

MicroST purchased the *BacLight* assays for bacterial cell viability. The Live/Dead tests for bacterial viability can be completed in 15 minutes and do not require wash steps. The Live cells fluoresce green while the Dead cells fluoresce red. The Live/Dead *BacLight* Kits can be used with an Epifluorescence microscope to distinguish live and dead bacteria in a mixed population simultaneously. The assays can discriminate as few as 1% to 10% live or dead cells in a population.

The bacterial viability can be measured in response to exposure to an ionized gas. The Live/Dead *BacLight* Bacterial Viability Kit provides sensitive, single step, fluorescent-based assays for bacterial cell viability. The Live/Dead Kit employs two nucleic acid stains:

1. the green-fluorescent SYTO-9 stain and the
2. red-fluorescent propidium iodide stain.

These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO-9 stain labels both live and dead bacterial cells. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO-9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red as illustrated in Figure 8.



**Figure 8.** Viability Analysis of Bacterial Suspensions comprising various Proportions of Live and Dead Bacteria. Live bacteria are stained fluorescent green (G) by SYTO 9 Stain and Dead bacteria are stained fluorescent red (R) by Propidium iodide.

#### OPTICAL COUNTING OF VIABLE AND DEAD BACTERIA

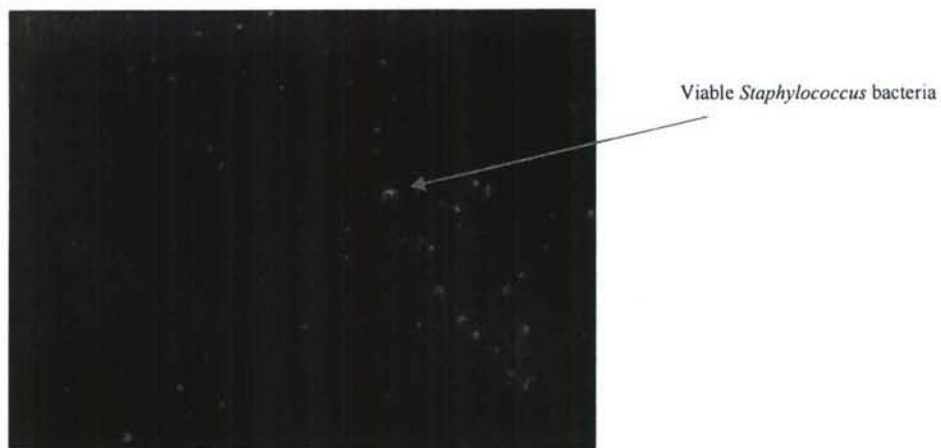
To observe the viable and dead bacteria with stains, MicroST has purchased an epifluorescent microscope as shown in Figure 9. The fluorescent signatures allow the bacterial stains to be illuminated and identified by software as particles. The individual particles can be directly observed and counted. The significance of this technique is that it enables particle counting at low particle concentrations for trace analysis. Therefore, we will record visual images using the new microscope and quantify results using the software-based particle counting process.



**Figure 9.** Fluorescent Microscope used to count particles on Microarrays

#### TESTING RESULTS WITH IONIZED GAS EXPOSURE TO BACTERIA

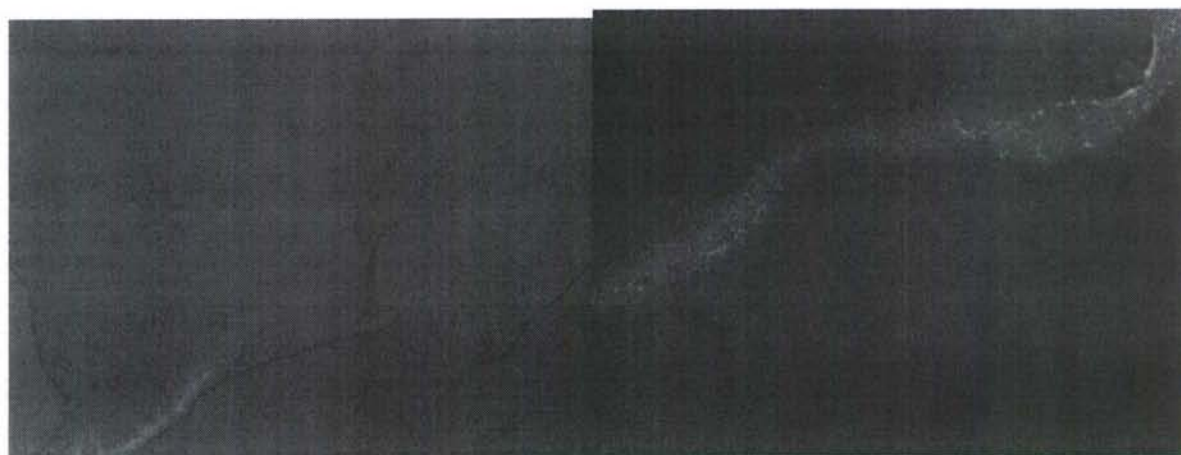
As illustrated in Figure 10, *Staphylococcus* bacteria were placed on a coupon and stained with fluorescent green (G) SYTO-9 to reveal live bacteria. This is an example of what the coupons looked like before plasma lysis treatment.



**Figure 10.** Viability Analysis of *Staphylococcus*. - Live bacteria are stained fluorescent green (G) by SYTO-9 Stain

#### 40 SECONDS OF PLASMA TREATMENT: OPTICAL "LIVE VERSUS DEAD"

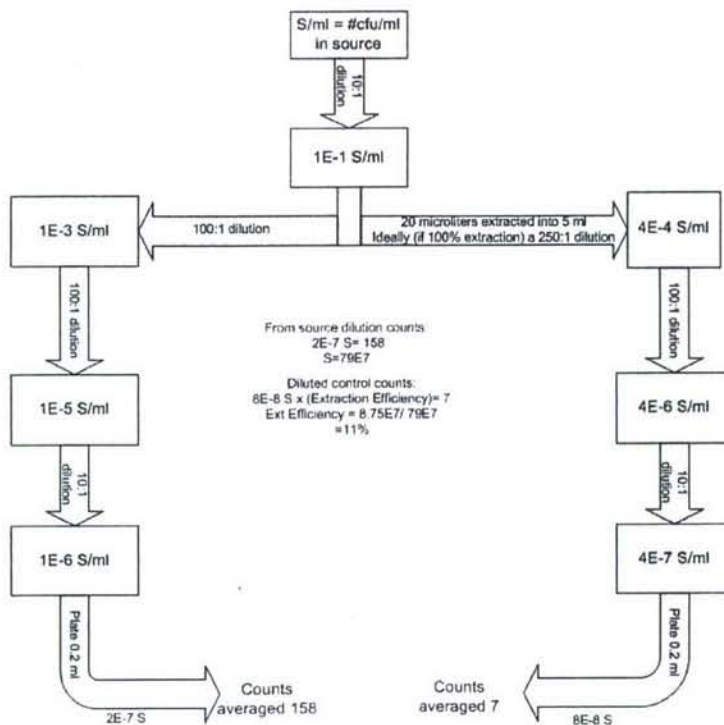
As shown in Figure 11, the Live (SYTO-9 green fluorescent) stain indicates negligible survival of *Staphylococcus* bacteria after 40 seconds of plasma treatment. The bright fluorescent red spots are indicative that the *Staphylococcus* bacteria present have been killed by the plasma treatment.



**Figure 11.** Viability Analysis of Staph. In the same area - Live bacteria are stained fluorescent green (G) by SYTO-9 Stain (left) and Dead bacteria are stained fluorescent red (R) by Propidium iodide (right). All of the bacteria stained are dead.

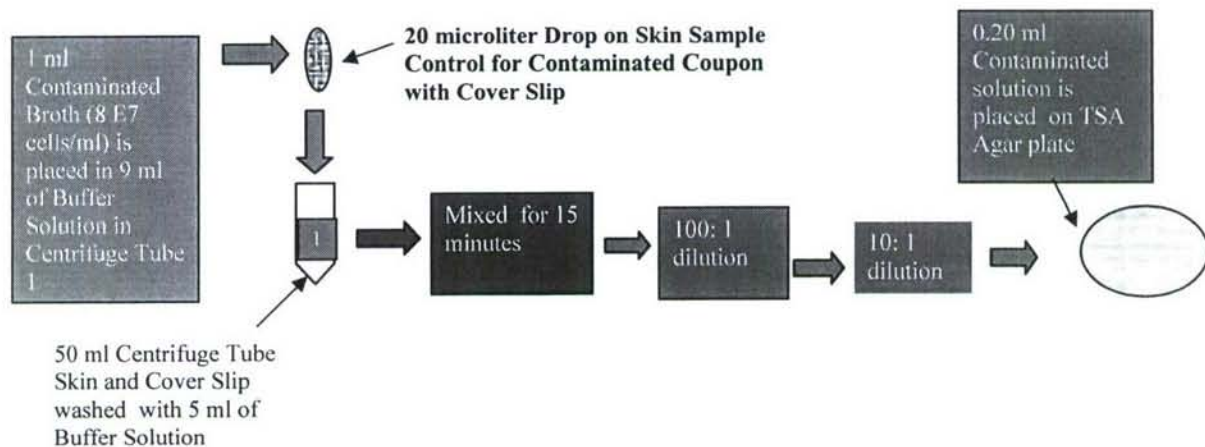
#### EXTRACTION EFFICIENCY DETERMINATION FOR BIOMATERIALS FROM A CONTAMINATED COUPON:

A contaminated coupon is placed under the plasma reactor for testing; however, the extraction efficiency determination uses a coupon contaminated with a drop of biomaterials being either bacterial spores or *Staph* cells (see Figure12).



**Figure 12.** Broth Concentration determination and Extraction Procedure for a Single Contaminated Skin Coupon

For determination of the extraction efficiency, the contaminated skin coupon (with 20 microliter of contaminated solution) is placed in a solution of approximately 9 milliliters of fluid. A schematic of this extraction process illustrates that the extraction samples are produced for each coupon and is shown schematically in Figure 13. One sample of each biomaterial and placed within less than 9 milliliter of sterilized water in a 50-milliliter centrifuge tube. Each coupon extraction sample was mixed for 15 minutes before undergoing serial dilutions. The skin coupon (with original cover slip) is washed above the centrifuge tube with 5 milliliter of water rinse from a pipette. The coupon is placed in a second dilution and mixed again. Similarly, 20 microliters are placed on an agar plate and incubated. Therefore, each coupon produces a primary plate and several dilutions.



**Figure 13.** Extraction Procedure for a Single Contaminated Coupon.



Four samples for each plasma condition and four controls were run with *Staphylococcus epidermis* on Testskin. For each sample, the skin and coverslip were washed in isotonic salt solution.

Estimating the source concentration from previous runs indicated that the control plates would likely be in the  $1E4$ - $1E5$  range, too high to count. So the control wash solutions (which should have the same number of organisms as the plasma-treated wash solutions, just alive in the case of the controls instead of dead in the case of sterilized samples) were diluted  $1E-3$  (one 100:1 dilution and one 10:1 dilution) and plated. The results averaged 7 counts per plate (actual results on four plates: 9,6,7,6). This means that if the control solution with the same concentration as the plasma-treated solution were plated, it would have yielded ~7000 colonies. The samples that were plasma treated for 90 seconds showed no growth on any of the four plates, indicating at least 99.9% sterilization as shown in Table 2.

**Table 2.** Plasma Sterilization of *Staph* as a Function of Time

| Test # | Time (sec.) | Plasma observations | Plate counts (0.2 ml of test concentrations) |
|--------|-------------|---------------------|----------------------------------------------|
| 1      | 60          |                     | 0                                            |
| 2      | 60          |                     | 54                                           |
| 3      | 60          |                     | 0                                            |
| 4      | 60          |                     | 0                                            |
| 5      | 90          |                     | 0                                            |
| 6      | 90          |                     | 0                                            |
| 7      | 90          |                     | 0                                            |
| 8      | 90          |                     | 0                                            |

#### Experimental Error Analysis Discussion

We use the plate counts from (E6 or E7) dilutions of the source to calculate the source concentration. We choose the dilution that gives us E2 counts, since that is the highest order of magnitude for which it is possible to count each colony (automated marking of colonies is followed by manual adjustments to ensure all colonies are counted), and for which the crowding of colonies does not cause growth anomalies. When ANOVA was run on 10 plates from three parallel dilutions of the same source (labeled S3C-A, S3C-B and S3C-C below), the result was that none of the three dilutions were statistically different. This confirmed that our dilution and plating procedures give reproducible results.



|         | S3C-A | S3C-B | S3C-C |
|---------|-------|-------|-------|
| Plate 1 | 578   | 536   | 529   |
| Plate 2 | 559   | 577   | 570   |
| Plate 3 | 539   | 609   | 532   |
| Plate 4 |       |       | 602   |

Anova: Single Factor

#### SUMMARY

| Groups | Count | Sum  | Average  | Variance |
|--------|-------|------|----------|----------|
| S3C-A  | 3     | 1676 | 558.6667 | 380.3333 |
| S3C-B  | 3     | 1722 | 574      | 1339     |
| S3C-C  | 4     | 2233 | 558.25   | 1198.917 |

#### ANOVA

| Source of Variation | SS       | df | MS       | F        | P-value  | F crit   |
|---------------------|----------|----|----------|----------|----------|----------|
| Between Groups      | 509.4833 | 2  | 254.7417 | 0.253459 | 0.782938 | 4.737414 |
| Within Groups       | 7035.417 | 7  | 1005.06  |          |          |          |
| Total               | 7544.9   | 9  |          |          |          |          |

The Analysis Toolpack in Excel was used to perform the data analysis ANOVA (analysis of variance). This analysis tool is used to test the null hypothesis Group A = Group B = Group C. The result of this operation is expressed in the value of F, which gets compared to a critical value of F (F crit) at the 0.05 level of significance. Because our computed test statistic F = 0.25 is less than F crit = 4.74, the null hypothesis is accepted. We conclude that there is no evidence of significant difference in the number of colony forming units between the three dilutions.

We enumerate below the propagation of errors through the multiple dilution steps; there are errors in each step based on estimated reading errors of pipetting. The final uncertainty is reported with the calculated source concentration.



### Calculation of S. epidermis Source Strength

After incubating the inoculated plates, the number of colonies on each plate were counted. Average, standard deviation, and standard error were calculated using Excel statistical functions.

0.200 ± 0.002mL of our 1E-6 dilution of the S. epidermis source was used to inoculate each plate. To determine the concentration of colony forming units in our 1E-6 dilution, we divide the plate counts by the volume of solution used, propagating error from our counts (std err) and error in pipetting.

The dilution formula  $\text{Conc}(i) \cdot \text{Vol}(i) = \text{Conc}(f) \cdot \text{Vol}(f)$  is used in conjunction with our previous calculation of the 1E-6 dilution concentration (our  $\text{Conc}(f)$ ), our final volume (25.0±0.1ml), and the volume (0.250±0.004ml) of the aliquot from the 1E-4 dilution to calculate the concentration of 1E-4 dilution. Error from pipetting is propagated with each operation.

The dilution formula is again used to calculate the concentration of the 1E-2 dilution, using the concentration of the 1E-4 dilution, the final volume of the dilution, and the volume of the aliquot from the 1E-2 dilution. Error from pipetting is propagated with each operation.

The dilution formula is again used to calculate the concentration of the S. epidermis source, using the concentration of the 1E-2 dilution, the final volume of the dilution, and the volume of the aliquot from the S. epidermis source. Error from pipetting is propagated with each operation.

Error Propagation with Multiplication:  
If  $g = g(y,t) = y \cdot t$   
then:

$$\delta_g = \sqrt{\delta_{gt}^2 + \delta_{gt}^2} = \sqrt{y^2 \delta_t^2 + t^2 \delta_y^2}$$

Error Propagation with Division:  
If  $g = g(y,t) = y/t$   
then:

$$\delta_g = \sqrt{\delta_{gt}^2 + \delta_{gt}^2} = \sqrt{\left(\frac{y}{t^2}\right)^2 \delta_t^2 + \left(\frac{1}{t}\right)^2 \delta_y^2}$$

### Error Propagation (from plating, back through dilutions, to source concentration determination)

#### 10<sup>-6</sup> Plate Counts: S3C-Dilution A

|         |     |
|---------|-----|
| Plate 1 | 578 |
| Plate 2 | 559 |
| Plate 3 | 539 |
| Ave     | 559 |
| Std Dev | 16  |
| Std Err | 9   |

| Plating:                 | counts/0.2mL |        |   |           |
|--------------------------|--------------|--------|---|-----------|
|                          | Value        | Error  | 9 | Rel. Err. |
| Counts                   | 5.59E+02     |        |   | 0.016     |
| / 0.2mL                  | 0.200        | 0.002  |   | 0.010     |
| = 1E-6 dilution (cfu/ml) | 2.79E+03     | 5.E+01 |   | 0.019     |

#### 3rd dilution:

|                          | Value    | Error  |  | Rel. Err. |
|--------------------------|----------|--------|--|-----------|
| 1E-6 dilution (cfu/ml)   | 2.79E+03 | 5.E+01 |  | 0.019     |
| * Final Volume (ml)      | 25.0     | 0.1    |  | 0.004     |
| = cfu in aliquot         | 6.98E+04 | 1.E+03 |  | 0.019     |
| / vol. of aliquot (ml)   | 0.250    | 0.004  |  | 0.016     |
| = 1E-4 dilution (cfu/ml) | 2.79E+05 | 7.E+03 |  | 0.025     |

#### 2nd dilution:

|                          | Value    | Error  |  | Rel. Err. |
|--------------------------|----------|--------|--|-----------|
| 1E-4 dilution (cfu/ml)   | 2.79E+05 | 7.E+03 |  | 0.025     |
| * Final Volume (ml)      | 25.0     | 0.1    |  | 0.004     |
| = cfu in aliquot         | 6.98E+06 | 2.E+05 |  | 0.025     |
| / vol. of aliquot (ml)   | 0.250    | 0.004  |  | 0.016     |
| = 1E-2 dilution (cfu/ml) | 2.79E+07 | 8.E+05 |  | 0.030     |

#### 1st dilution:

|                        | Value    | Error  |  | Rel. Err. |
|------------------------|----------|--------|--|-----------|
| 1E-2 dilution (cfu/ml) | 2.79E+07 | 8.E+05 |  | 0.030     |
| * Final Volume (ml)    | 25.0     | 0.1    |  | 0.004     |
| = cfu in aliquot       | 6.98E+08 | 2.E+07 |  | 0.030     |
| / vol. of aliquot (ml) | 0.250    | 0.004  |  | 0.016     |

|                         |         |        |  |       |
|-------------------------|---------|--------|--|-------|
| = S. e. Source (cfu/ml) | 2.8E+09 | 1.E+08 |  | 0.034 |
|-------------------------|---------|--------|--|-------|

#### Source

| Concentration: | Value   | Error  |  | Rel. Err. |
|----------------|---------|--------|--|-----------|
| (cfu/ml)       | 2.8E+09 | 1.E+08 |  | 0.034     |

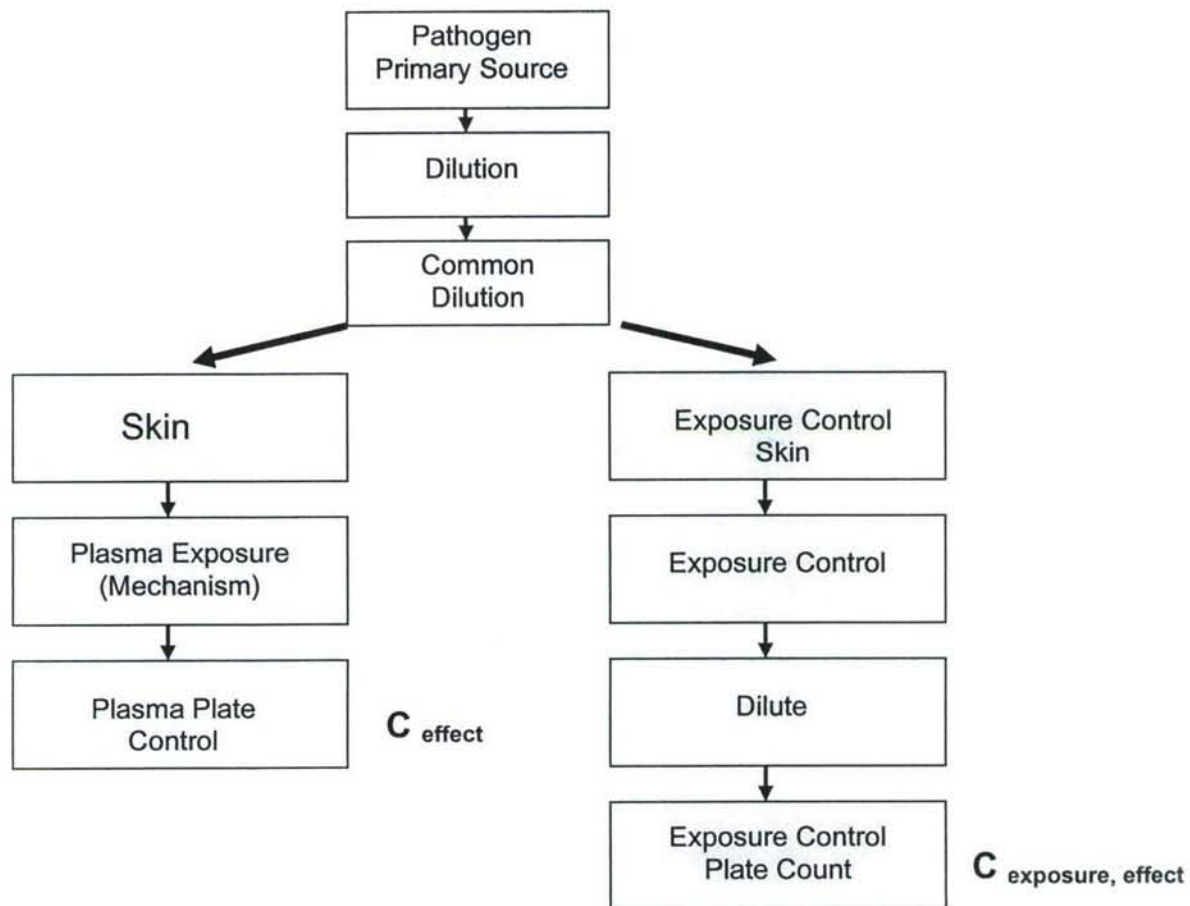
## Error Propagation of parallel dilutions

| 10 <sup>-6</sup> (-6) Plate Counts: S3C-Dilution B |  |              |        |           | 10 <sup>-6</sup> (-6) Plate Counts: S3C-Dilution C |  |              |        |           |
|----------------------------------------------------|--|--------------|--------|-----------|----------------------------------------------------|--|--------------|--------|-----------|
| Plate 1                                            |  |              |        | 536       | Plate 1                                            |  |              |        | 529       |
| Plate 2                                            |  |              |        | 577       | Plate 2                                            |  |              |        | 570       |
| Plate 3                                            |  |              |        | 609       | Plate 3                                            |  |              |        | 532       |
| Ave                                                |  |              |        | 574       | Plate 4                                            |  |              |        | 602       |
| Std Dev                                            |  |              |        | 30        | Ave                                                |  |              |        | 558       |
| Std Err                                            |  |              |        | 17        | Std Dev                                            |  |              |        | 35        |
| Std Err                                            |  |              |        | 17        | Std Err                                            |  |              |        | 17        |
| Plating:                                           |  |              |        |           | Plating:                                           |  |              |        |           |
|                                                    |  | counts/0.2mL |        |           |                                                    |  | counts/0.2mL |        |           |
|                                                    |  | Value        | Error  | Rel. Err. |                                                    |  | Value        | Error  | Rel. Err. |
| Counts                                             |  | 5.74E+02     | 17     | 0.030     | Counts                                             |  | 5.58E+02     | 17     | 0.031     |
| / 0.2mL                                            |  | 0.200        | 0.002  | 0.010     | / 0.2mL                                            |  | 0.200        | 0.002  | 0.010     |
| = 1E-6 dilution (cfu/ml)                           |  | 2.87E+03     | 9.E+01 | 0.032     | = 1E-6 dilution (cfu/ml)                           |  | 2.79E+03     | 9.E+01 | 0.033     |
| 3rd dilution:                                      |  |              |        |           | 3rd dilution:                                      |  |              |        |           |
|                                                    |  | Value        | Error  | Rel. Err. |                                                    |  | Value        | Error  | Rel. Err. |
| 1E-6 dilution (cfu/ml)                             |  | 2.87E+03     | 9.E+01 | 0.032     | 1E-6 dilution (cfu/ml)                             |  | 2.79E+03     | 9.E+01 | 0.033     |
| * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     | * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     |
| = cfu in aliquot                                   |  | 7.18E+04     | 2.E+03 | 0.032     | = cfu in aliquot                                   |  | 6.98E+04     | 2.E+03 | 0.033     |
| / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     | / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     |
| = 1E-4 dilution (cfu/ml)                           |  | 2.87E+05     | 1.E+04 | 0.036     | = 1E-4 dilution (cfu/ml)                           |  | 2.79E+05     | 1.E+04 | 0.037     |
| 2nd dilution:                                      |  |              |        |           | 2nd dilution:                                      |  |              |        |           |
|                                                    |  | Value        | Error  | Rel. Err. |                                                    |  | Value        | Error  | Rel. Err. |
| 1E-4 dilution (cfu/ml)                             |  | 2.87E+05     | 1.E+04 | 0.036     | 1E-4 dilution (cfu/ml)                             |  | 2.79E+05     | 1.E+04 | 0.037     |
| * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     | * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     |
| = cfu in aliquot                                   |  | 7.18E+06     | 3.E+05 | 0.036     | = cfu in aliquot                                   |  | 6.98E+06     | 3.E+05 | 0.037     |
| / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     | / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     |
| = 1E-2 dilution (cfu/ml)                           |  | 2.87E+07     | 1.E+06 | 0.039     | = 1E-2 dilution (cfu/ml)                           |  | 2.79E+07     | 1.E+06 | 0.040     |
| 1st dilution:                                      |  |              |        |           | 1st dilution:                                      |  |              |        |           |
|                                                    |  | Value        | Error  | Rel. Err. |                                                    |  | Value        | Error  | Rel. Err. |
| 1E-2 dilution (cfu/ml)                             |  | 2.87E+07     | 1.E+06 | 0.039     | 1E-2 dilution (cfu/ml)                             |  | 2.79E+07     | 1.E+06 | 0.040     |
| * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     | * Final Volume (ml)                                |  | 25.0         | 0.1    | 0.004     |
| = cfu in aliquot                                   |  | 7.18E+08     | 3.E+07 | 0.040     | = cfu in aliquot                                   |  | 6.98E+08     | 3.E+07 | 0.040     |
| / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     | / vol. of aliquot (ml)                             |  | 0.250        | 0.004  | 0.016     |
| = S. e. Source (cfu/ml)                            |  | 2.9E+09      | 1.E+08 | 0.043     | = S. e. Source (cfu/ml)                            |  | 2.8E+09      | 1.E+08 | 0.043     |
| Source Concentration:                              |  |              |        |           | Source Concentration:                              |  |              |        |           |
| (cfu/ml)                                           |  | 2.9E+09      | 1.E+08 | 0.043     | (cfu/ml)                                           |  | 2.8E+09      | 1.E+08 | 0.043     |

This source concentration is used in the calculation of how many colony forming units (CFUs) of *Staphylococcus epidermis* are deposited on the skin samples for the plasma experiments. The remaining operations in the experimental path will each be examined for errors.

## SUMMARY OF EXTRACTION AND CULTURING PROCEDURES

The extraction and culturing of the contaminated skin samples is shown in Figure 14. These labels were consistently used to report data.



**Figure 14.** Summary of Control and Treatment Procedures

#### CULTURING APPROACH FOR QUANTITATION OF PLASMA STERILIZATION EFFICIENCY

To minimize error in the amount of inoculant delivered to the skin samples, we are now using a micro-syringe pipette with disposable sterile tips. The syringe is set to draw 2 microliter drops repeatedly; blow-out ensures the entire volume is delivered. An additional benefit to this small volume is better containment of the bacteria to the skin, eliminating spreading to the glass slide. This ensures uniform plasma treatment and minimizes losses due to bacteria adhering to the glass. We are in the process of experimentally testing the reproducibility of the CFUs delivered per drop by the micro-syringe pipette. The droplets are too small to use weight as a measure of reproducibility of droplet size with enough accuracy (our lab scale reads tenths of mg, so variation in its last digit represents 5-10% of the total weight). Instead, we deliver the 2-microliter drop directly to 2 ml wash water (isotonic salt solution), dilute and plate. We will also compare these results to those of processed exposure-control samples, thereby measuring the efficiency of extraction of *Staph. epidermis* from the skin by our wash procedure. We can calculate the error in the dilutions of the exposure controls. The final error is the plating error, which was shown to be ~3% in the analysis of the source dilutions (shown above).

The errors in determining percent reduction of bacteria associated with the wet lab processing of plasma-treated samples include the experimental errors in the source amount delivered to skin, extraction efficiency from skin, subsequent dilutions (of exposure controls) and plating. We have shown calculations and/or data on the errors in plating and dilutions, and the propagation of these errors in

determining our source concentration. We have upgraded our source delivery apparatus to lower errors in the amount of source delivered; we are in the process of measuring these errors.

With the smaller amount of water on the skin samples, it is possible the plasma will dry the skin during treatment. To investigate the effect of this, we have run an initial experiment of exposure controls comparing extraction of freshly-inoculated skin with skin that was allowed to dry for a couple of hours (no visible water on the surface of the skin), and saw no statistical difference in the data sets. The plate counts were in the E1 instead of the desired E2 range, so we will repeat the experiment with a stronger source. (The source concentration is dynamic, and can drop off if nutrients run low; it is difficult to keep in stasis without affecting its growth response. This is one reason we always run a source dilution in parallel with the experiments.)

The error in plasma treatment can be mostly ascribed to systematic error in the plasma test stand. To measure this requires development of sophisticated electronic diagnostics, which is being undertaken.

### Experimental Results

The main experimental conditions sought are: to produce uniform skin samples for plasma treatment that have a high enough bacterial concentration to show log 3 reduction in population after extraction, to find plasma conditions that do not damage the skin but produce the needed log 3 reduction (without killing all the bacteria, so reproducibly few are left to plate).

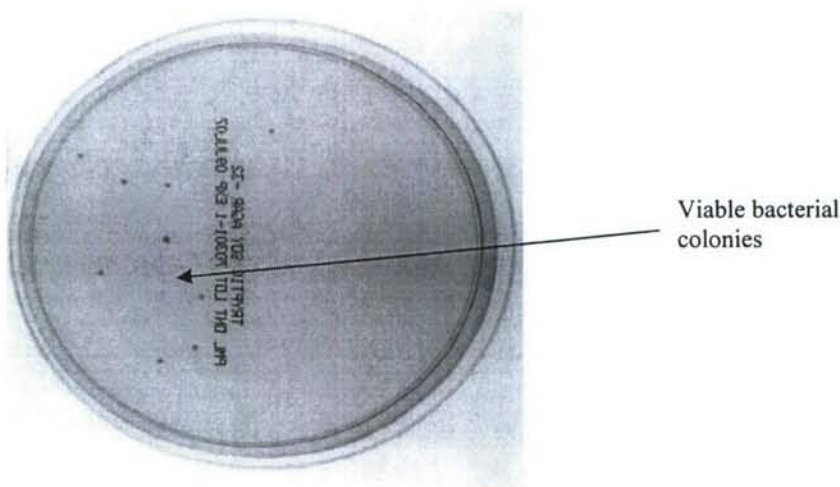
Initial experiments in December showed a high kill rate of bacteria, but micro-arcing in the plasma resulted in damage to the skin samples. Exposure time of the samples was reduced gradually from 270 seconds to 180, 90, 60, and then 30 seconds. There were still unpredictable micro-arcing events. To achieve a more uniform glow discharge, a thin, flat barrier dielectric was added to the upper electrode. The distance between electrodes needed to be re-optimized, and sample exposure times re-evaluated. The next several experiments showed high kill rates at 60, 45 and 30 seconds, but in each experiment there was an outlier that did not show as high a kill rate as the others exposed to the plasma for the same treatment time. Cleaning procedures were increased, in case there was a contamination problem, but it did not solve the issue. It was concluded that the droplet of bacterial solution, delivered to the skin samples, was too large and caused non-uniform treatment, due in part to spreading of the solution outside the electrode area. To improve the procedure, a micro-syringe pipette capable of repeatably delivering several-micro-liters was selected.

Initial evaluation indicates that the smaller droplet size of the new micro-syringe pipette, in addition to being more accurate and repeatable for source delivery, also improves the uniformity of plasma treatment. By allowing the droplet to spread out on the skin without any overflow to the glass substrate, the bacterial solution contained on the skin is more uniformly exposed to the plasma. Also, using smaller droplets avoids wetting the cover slip used with each experiment, thereby simplifying the extraction procedure. This should reduce the variation in extraction and increase the extraction efficiency, due to reduction in losses from bacteria adhering to the glass. Preliminary results indicate an extraction efficiency increase from ~10% to ~40% with the new procedure.

Each skin sample is contaminated with a 2-microliter drop of biomaterial. At the source concentrations used, this yields  $\sim 10^6$  *Staphylococcus* that spreads over a fraction of a square centimeter of skin sample. Using a smaller droplet size reduces the amount of bacteria delivered to the skin samples, but two factors bring the concentration back to the range needed to clearly show log 3 reduction. We have boosted the concentration of the source by more frequent cycling of air and fresh nutrient broth. Also, the increase in extraction efficiency increases the number of bacteria available in the exposure controls and the plasma-treated samples, so we can find the conditions that give E1 colonies plated from the plasma-treated

samples, and E2 from E3-diluted controls (a total of E5 on the controls compared to E1 on the plasma samples would show a log 4 reduction).

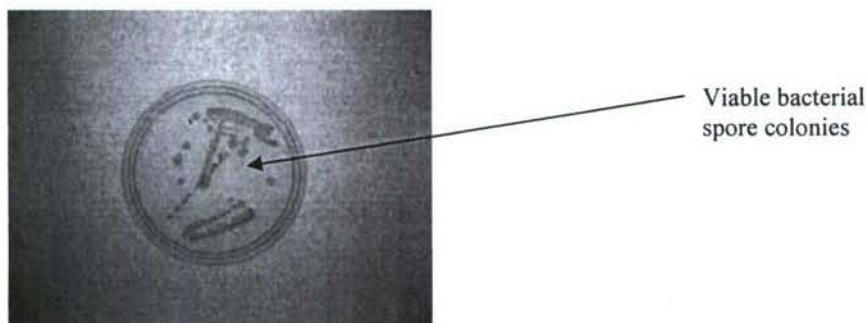
We have succeeded in finding the desired conditions, and are ready to run a full series of tests (see Figure 15). Using the current test stand setup, 15 seconds of plasma treatment shows on the order of E1 colonies, compared to ~ E5 from the exposure controls. The kill threshold seemed fairly sharp, between 10 and 15 seconds plasma exposure. See the image below for a representative sample of directly plated wash water (undiluted) from a plasma-treated sample. The 15-second-treated sample shows very few viable organisms left. Now that we have found the plasma treatment time that will give us the desired statistics to show log3 reduction, we will run repeated experiments at these conditions.



**Figure 15.** Finding the kill threshold for *Staphylococcus*. At a 15 second exposure, the plasma-treated sample showed very few organisms left. The E2 dilution of the exposure control grew E3 colonies.

#### CULTURING APPROACH: TESTING RESULTS WITH IONIZED GAS EXPOSURE TO BACTERIAL SPORES

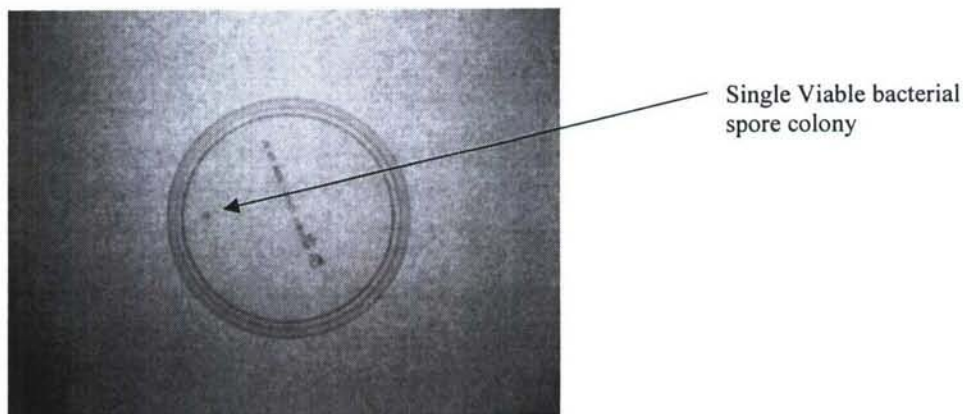
As illustrated in Figure 16, bacterial spores, *Bacillus subtilis var niger*, were placed on a coupon, contaminated with bacterial spores, sterile water extracted, and grown on petrie dishes filled with growth media. This is an example of what the control sample looks like after incubation at 30°C. These screening tests illustrate the trends that one can expect to find as quantitative tests are performed. The approach is to test the hypothesis of very short plasma exposure times to initiate germination of the bacterial spores. After a 10 minute period, the plasma will be reinitiated to deactivate the bacterial spores.



**Figure 16.** Viability Analysis of Bacterial Spores

### FIVE MINUTES OF PLASMA TREATMENT FOR BACTERIAL SPORES

As shown in Figure 17, few spores survive several minutes of plasma treatment. The live colonies on the growth media indicate that the bacterial spore survived the plasma process. The single colony indicates negligible survival of millions of bacterial spores after 5 minutes of plasma treatment. The applied power was 15 watts applied to about 10 cm<sup>2</sup>. Quantitative results of bacterial spore will be forthcoming and presented in November at DARPA.



**Figure 17.** Viability Analysis of Bacterial Spores after several minutes of Plasma Treatment.

### Task 3. Determine Mechanism of Ionized Gas Effect: Vary Carrier Gases

To isolate the decontaminating effects of the plasma blanket unit, carrier gases such as air, nitrogen, and oxygen/nitrogen mixtures will treat surfaces contaminated with biological simulants and the inoculant survival rate will be measured.

### DETERMINE EFFICIENCY OF INOCULANT SURVIVAL AS A FUNCTION OF ELECTRICAL FIELD CONDITIONS

Researchers such as Schoenbach have noted that vegetative cells readily lyse under intense electric field conditions but spores and viruses retain their cellular integrity under high voltage conditions. Intense electric field treatment did not reduce the germination of BC spores [Birmingham 2002]. The electroporation destruction of cells is believed to be due to the disruption of the cell membrane functions. In these experiments, intense electric fields will be applied to contaminated artificial skin coupons slightly below the corona onset-voltage to investigate the impact of electric field on the biological materials survival. The ionized gas sterilization unit will be tested under various operating conditions and the appropriate variables will be analyzed.

Plasma reactors can be classified by their physical construction and method of coupling energy. Each physical arrangement has certain advantages for reactions, while the energy is closely coupled with the reactor design. The plasma reactor behaves electrically as a capacitor, C, (albeit a 180° flat capacitor) while the secondary windings on the transformer behave as an inductor, L. Therefore, the resonant frequency, F, of the system can be represented by Equation (1):

$$F = \frac{1}{2\pi} \sqrt{\frac{1}{LC}} \quad (1)$$

While the inductance of the system will be fixed with the choice of transformer, the capacitance of the system may vary as objects are placed therein for sterilization. One can find the capacitance of the reactor by considering the equivalent capacitance (in series) in Equation (2):

$$C = \frac{1}{\left(\frac{1}{C_d}\right) + \left(\frac{1}{C_m}\right) + \left(\frac{1}{C_a}\right)} \quad (2)$$

where  $C_d$  is the capacitance of the dielectric barrier,  $C_m$  is the capacitance of the material to be sterilized (either dielectric or metallic), and  $C_a$  is the capacitance of the air. It should be noted that the capacitance of the reactor changes tremendously when the ionized gas plasma fills the gap. However, we are solving for the corona-onset conditions.

It can be seen that the main contribution to the capacitance of the system is the dielectric on the electrode, and not the object within the plasma region. These results have been verified experimentally. Since the capacitance of the system is related to the applied voltage,  $V$ , solving for the capacitive voltage drop across the dielectric barrier,  $V_1$ , can be seen in Equation (3):

$$C = \frac{q}{V} \rightarrow V_1 = \frac{V(C_m C_a)}{C_m C_a + C_m C_d + C_d C_a} \quad (3)$$

As shown in Equation (3), the presence of the dielectric on the electrodes creates a voltage drop for the initiation of the plasma. Similarly, the voltage drop reveals that the electric field strength of 30 kilovolts per centimeter of corona-onset potential in air can be achieved more easily with a material having high effective capacitance. Counter to intuition, the placement of various objects within the plasma region enhances the corona production process. Further, using a miniaturized system where the electrodes are not placed very far apart implies that the voltages required to achieve 30 kV/cm can be on the order of 3000 volts or smaller. The capacitance of the plasma reactor and the artificial skin will be determined.

#### DETERMINE EFFICIENCY OF INOCULANT SURVIVAL AS A FUNCTION OF IONIZED GAS EXPOSURE TIME

The inoculant survival as a function of ionized gas exposure will be explored. Initially, the sample will be exposed for tens of seconds to the ionized gas. If the treatment is not effective with a minute of ionized gas treatment time, then a several minute exposure will be tested as the final test conditions. If time permits, the efficacy of intermittent and periodic exposures will also be considered.

#### DETERMINE EFFICIENCY OF INOCULANT SURVIVAL AS A FUNCTION OF DEPTH OF ARTIFICIAL SKIN

The key question that needs to be addressed during this study is whether the effect extends below the skin surface. The following initial set of experiments should reveal the depth of sterilization that ionized gas exposure provides. *Staphylococcus* and possibly *Leishmaniasis* bacteria will be deposited by syringe on the surface (0 mm) and alternately on the bottom of the artificial skin at 1 mm (and possibly 5 mm of depth). Experiments may be performed with artificial skin (e.g., Integra), cadaver skin, or animal skin of carefully controlled thickness.

#### DETERMINE EFFICIENCY OF INOCULANT SURVIVAL AS A FUNCTION OF DEPOSITED POWER

The electrical characteristics were initially described by Manley and the measurement circuit is shown in Figure 18. When the air dielectric breaks down, the discharge is limited by the buffer capacitance. The ionized gas places a uniform discharge over the dielectric surface of the artificial skin, the dielectric stores charge creating a reverse voltage that limits charge. This current cut-off exists until the applied voltage reverses to ionize the gap and place the opposite charge on the dielectric surface.

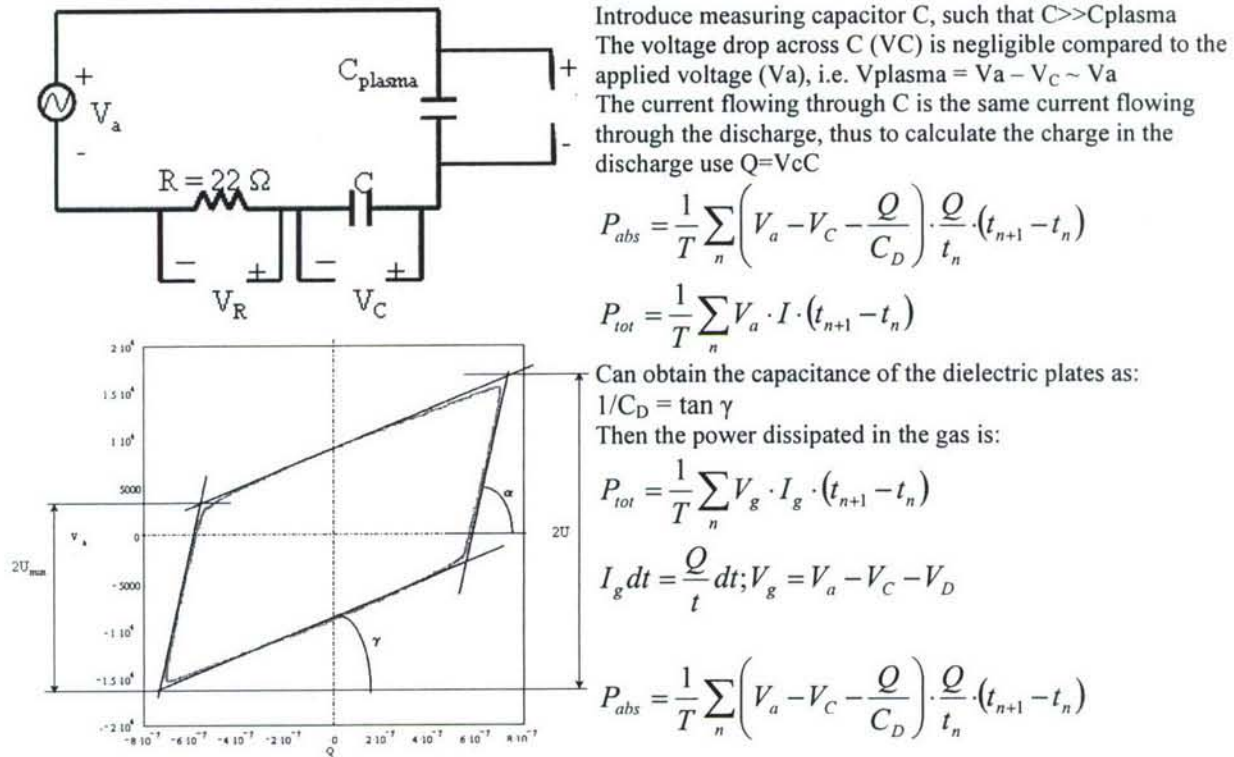
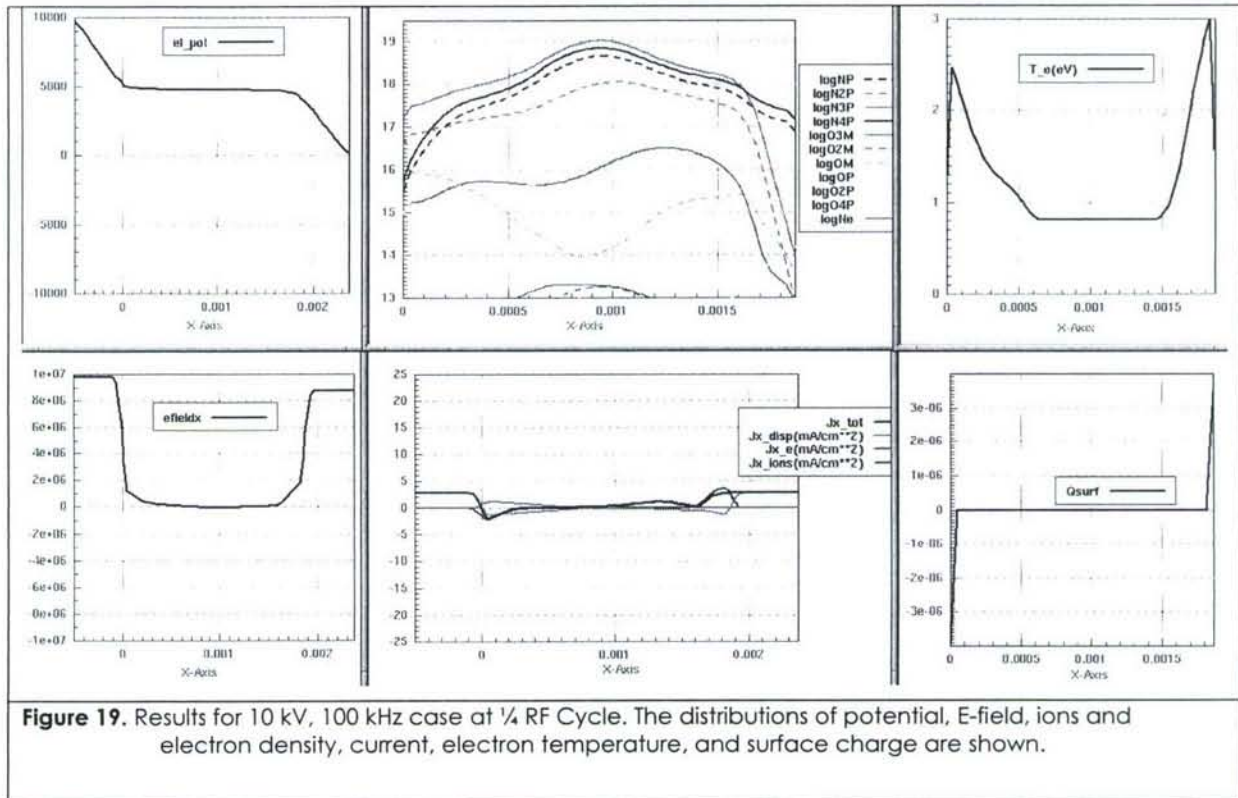


Figure 18. Equivalent Circuit to measure Electrical Character of Plasma Discharge

The dielectric barrier discharge plasma composition and dynamics in atmospheric air is simulated by combining the nitrogen and oxygen chemical mechanisms (as shown in Figure 19). The  $N_2$ - $O_2$  interaction steps of interest are initiated by electron impact with subsequent atom-molecule interactions. For example, steps 1-4 are dissociation of  $O_2$  (with the collision of  $N_2$  species) to become oxygen atoms. Steps 5-6 account for detachment of  $O_2^-$  by nitrogen species. The results for RF power of 10kW, 100 kHz are shown in Figure 19. In Figure 19, the electrostatic potential and electric field strength, ions and electron densities, conduction current, electron temperature, and surface charge at the interface of dielectric material and plasma, are plotted at  $1/4$ ,  $2/4$ ,  $3/4$  and  $4/4$  of one RF cycle. The major ion species are  $N_4^+$ ,  $N^+$ ,  $O_2^+$ ,  $O_3^+$ . The electron density is lower by at least one-order of magnitude than the major negative ion species of oxygen. It is worth pointing out that at the RF frequency of 100 kHz, not only the electrons, but also the much heavier ions species oscillate following the instantaneous electrostatic potential. The surface charge accumulation flips sign along with the RF voltage. Figure 19 shows the electron density (at  $x=1$  mm) for two RF cycles. It is seen that the periodical steady-state is reached in the simulations. The peak value of the electron density is about  $10^{+16}$  while the bottom value is about  $10^{+15} \text{ m}^{-3}$ .



### 3. Summary of Accomplishments

The goal of this project is to demonstrate a flexible plasma reactor to wrap around body parts that are infected and promote sterilization of skin. A need exists for a portable sterilization system that can quickly sterilize skin as well as medical instruments and skin catheters. To address this challenge, MicroStructure Technologies (MicroST) demonstrated a compact, low maintenance, and high efficiency plasma blanket that

- ❖ can kill pathogens efficaciously on biological surfaces without damage to the biological surface.
- ❖ kills the spores.
- ❖ Inoculant bacteria such as *Staphylococcus* and possibly *Leishmaniasis*.

Several measurements of plasma sterilization of *Staphylococcus epidermis* (Staph):

A. The sample extraction and sterilization efficiencies:

Extraction experiments illustrated the importance of using test skin to more accurately determine the removal of bacteria from the skin surface is 11%. The plasma sterilization efficiency was established at 99.9% currently.

B. The establishment of a correlation between optical measurements and plating was attempted without success. The ability to correlate the stain-assisted concentrations of cells with optical microscopy has been determined to be suspect. It was found that the stain suffered photon bleaching that perturbed the measurements.