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TITLE: Compressing Time and Space for an In Situ Dermal Graft Printing Paradigm

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14. ABSTRACT
Currently, no studies have investigated excised wound beds as a supportive matrix for a well-defined population of stem cells cultured directly within the wound bed niche. To capitalize on the idea of a wound bed-stem cell synergy, the discovery science phase of work in this proposal will rest on the premise that the native wound microenvironment will be the optimal bioreactor for a stem cell-based dermal graft model. Upon completion of the build of the high-resolution MEW process with implementation of a heat-exchange collector plate and establishing fundamental understanding of the collector temperature effect under stationary and single fiber printing modes of operation, optimization of design parameters for the 3D template printing were investigated in Year 2 of the project. The systematic design of experiments carried out in the current reporting period yielded significant results, including the quantitative evolution of scaffold morphology with layer number at different collector temperatures. Based on the PI team's established process testbed and understanding of process parameterization in Year 1-2 of the project, a major task for the next reporting period will be to extend the MSC biological phenotype characterization studies for longer duration time courses. Although early indications based on the MSC culture on MEW|0-90o experiments demonstrates an enhancement in preserving the stem cell phenotype during the first week of culture compared to the 2D controls, the time course will be extended (to two weeks or longer) in the next design of experiments in order to observe how long the stem cell marker expression is preserved, along with the degree of homogeneity in the 3D cultured stem cell population. Another major task for the next reporting period will be to investigate the effect of alternate porous microarchitectures (e.g. MEW|0-45o) on the resultant MSC phenotype. Finally, another significant task for the next reporting period will be to demonstrate in situ electrohydrodynamic printing of the multilayered fibrous graft model directly onto the phantom wound ulcer bed.

15. SUBJECT TERMS
Additive manufacturing, 3D printing, dermal graft, stem cell, wound, ulcer, immunomodulation, in situ printing

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1. INTRODUCTION:

The subject of this research is developing an innovative therapeutic strategy for chronic cutaneous wound care that is complicated by an active military and Veteran patient population with a heterogeneous ulcer etiology. Furthermore, sub-optimal patient outcomes with existing wound care technologies and approaches reflect the inadequacy of single, rudimentary agents to mediate the complex interplay of wound healing. Therefore, the purpose of this research is to develop an in-situ scalable therapeutic strategy that reliably harnesses stem cell regenerative and immunomodulatory properties that effectively reverse the debilitating consequences of chronic ulcer wound healing. The scope of this discovery phase of research will be the design and implementation of a precision, super-resolution printing process that yields microscale fiber-based templates to program MSC expansion and modify the wound bed niche towards subverting the chronic wound phenotype that is currently refractory to existing treatment modalities.

2. KEYWORDS:

Additive manufacturing, 3D printing, dermal graft, stem cell, wound, ulcer, immunomodulation, in situ printing

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project as stated in the approved SOW are partitioned into two Specific Aims: 1) Test the identified key process and material parameters with optimization for uniform mechanical stretching and alignment of printed fibers, and 2) Design, fabricate, and evaluate precision dermal graft model under varying microscale geometrical cues. For the associated two major tasks, the milestones and percentage of completion are as follows:

Milestone 1 Achieved (75% completion) at 8 months: Identification of process and material parameter regimes that enable fiber stretching and alignment in an in situ phantom wound bed.
Milestone 2 Achieved (50% completion) at 18 months: Production of a homogeneous self-renewing MSC population as a dermal graft model.

What was accomplished under these goals?

Major Activities:
In Year 3 of the project, the PI team completed the bioprinting of the phantom wound ulcer bed along with continued investigation into MSC phenotype characterization on the heterogeneous 3D structured biomaterial substrates.

A. Bioprinting of phantom wound ulcer bed
In this section, the direct bioprinting of hydrogel filament-based burn skin phantom onto a non-planar surface is accomplished. The non-planar surface is placed on the printer platform, and the bio-ink is directly deposited onto the wound region by implementing the continuous 3D non planar tool-path which is generated by the proposed algorithm. The ROI is the colored area which is a part of the whole 3D model mimicking the burn area. During the printing, 3D object is place statically within the printable area, the printing process could start automatically after the user picking up the needle position. The bioink is loaded within the extruder with cell seeded, from which the meshed structure could be printed in a layer by layer fashion. A total cell number of 2 × 106 /ml fibroblast cells are uniformly dispersed within the 2-layer tissue construct. The meshed structure has 2 mm intervals between filaments to form a porous structure which enables the delivery of nutrients and oxygen for maintaining cell viability and proliferation. Fig. 8 displays the printing process and the micro structure of the printed constructs. The meshed structure contains 2 layers of cross-linked filament. Fig 1. (a) shows the non-planar printing process and (b-d) characterize the printed outcome at mm scale, which depict the detailed 2D structure of the printed construct. A distinguishable multilayered meshed structure can be observed. The bioink has preferred properties for printing which ensures adequate shape fidelity and mechanical properties. Besides, the cell viability and proliferation are also study. Fig. 2 shows the printed constructed within a simulated phantom. Fig. 2 (b)-(d) show the detailed structure of printed mesh structure where the grid shape is maintained and the layer boundary is also distinguishable.

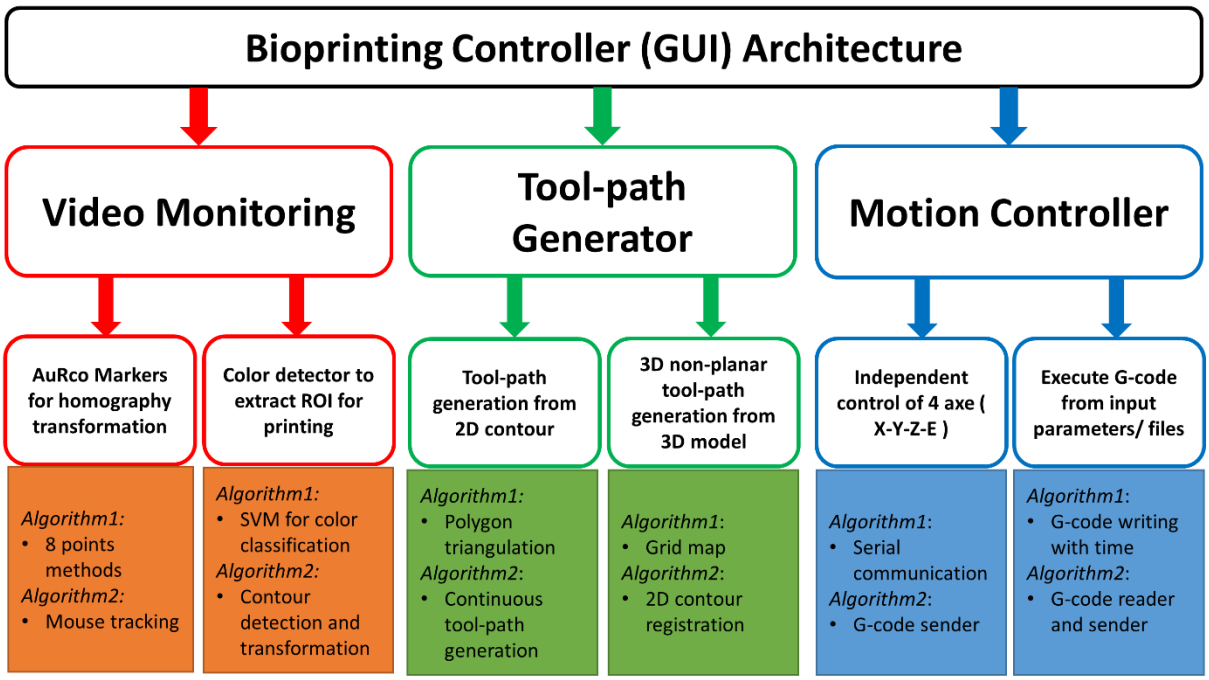

Figure 1. The 3D bioprinting process and micro-structure of multi-layer cell-laden hydrogel-based phantom skin wound bed onto a non-planar surface.

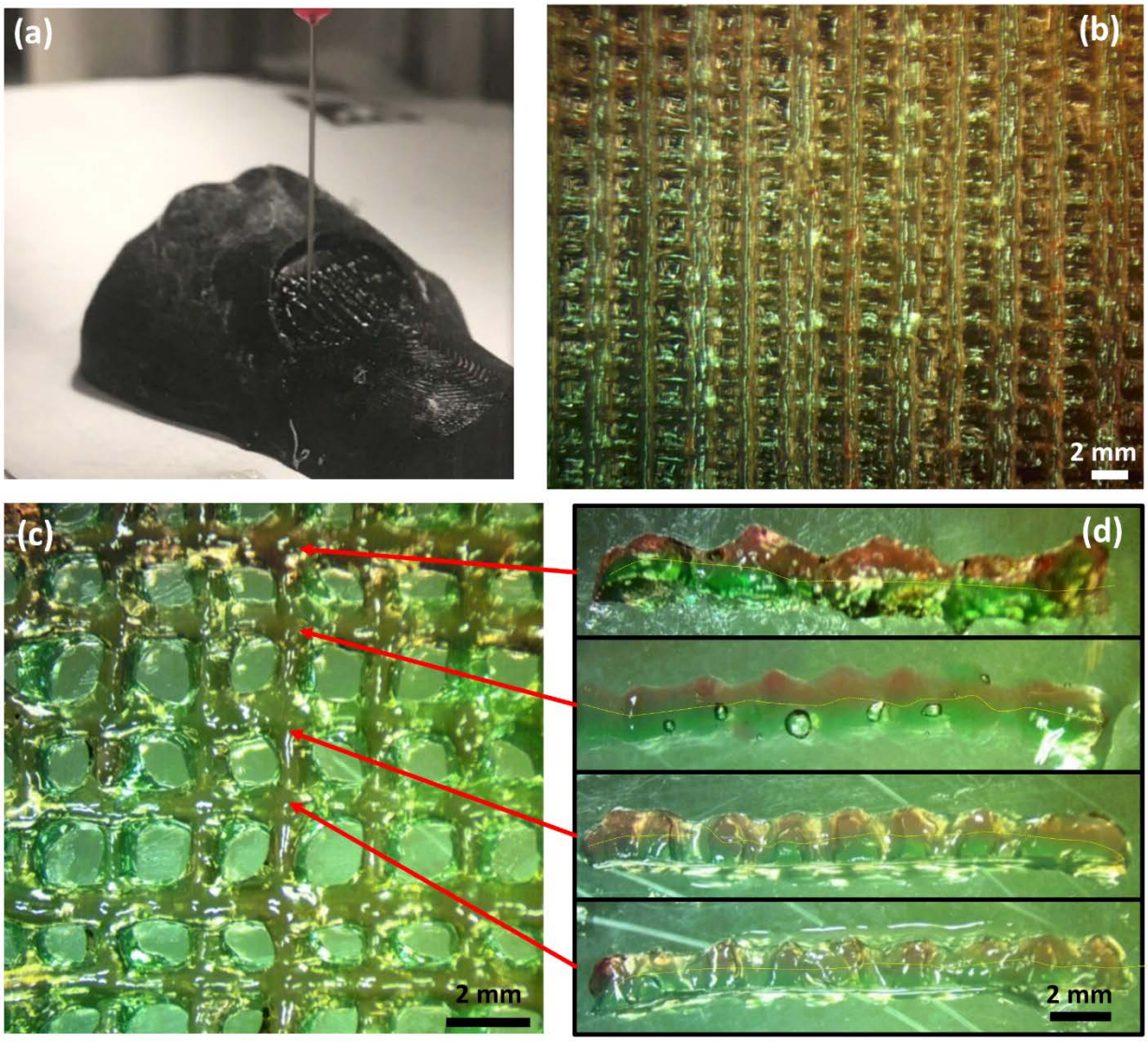

Figure 2. The printed phantom constructed. (b)-(d) show the detailed structure of printed mesh structure where the grid shape is maintained and the layer boundary is also distinguishable.

B. MSC Phenotype Characterization

In Year 2, it was demonstrated that observed that MSCs cultured on the MEW|0-90^{o} substrates remain viable and self-renew by populating the effective fiber adhesive area without losing their phenotype during the first week of culture. In Year 3, the time course of the experiment is extended to two weeks demonstrating that cells continued to proliferate without losing their stem cell marker expression (Figure 3). Then, it is tested whether an altered porous microarchitecture at under a 3D setting could result to differential MSCs phenotypes compared to the Controls and the MEW|0-90^{o} substrate at the prescribed time points. Furthermore, in Year 3, it is observed that MSCs maintain their sternness during the first week of culture on MEW|0-45^{o} substrates (Figure 4-A- O), while they lose their MSC phenotype sometime during the second week of culture (Figure 4-B-E).

In summary, in Year 3, the Pl team demonstrated that electrospinning can be combined with additive manufacturing for the fabrication of high fidelity biomaterial fibrous substrates with geometrical feature sizes at cell operating length scales. In addition to that, a machine learning-based metrology framework is advanced that can quantitatively assess and classify the effect of geometrical confinement on human adherent cells across different fibrous substrates dimensionalities and topographies. To measure this effect, a quantitative confocal imaging workflow is demonstrated revealing distinct confinement states characterized by less or more migratory early cell shape phenotypic responses both at the cellular and subcellular focal adhesion protein level. The technology platform established here serves as a major step towards the development of bioinformatics-guided additive manufacturing systems. The combination of advanced fabrication and metrology tools paves a new avenue for the systematic engineering of functional biomaterial systems that can reliably guide distinct, uniform and predictable stem cell responses for a wide range of biomedical applications.

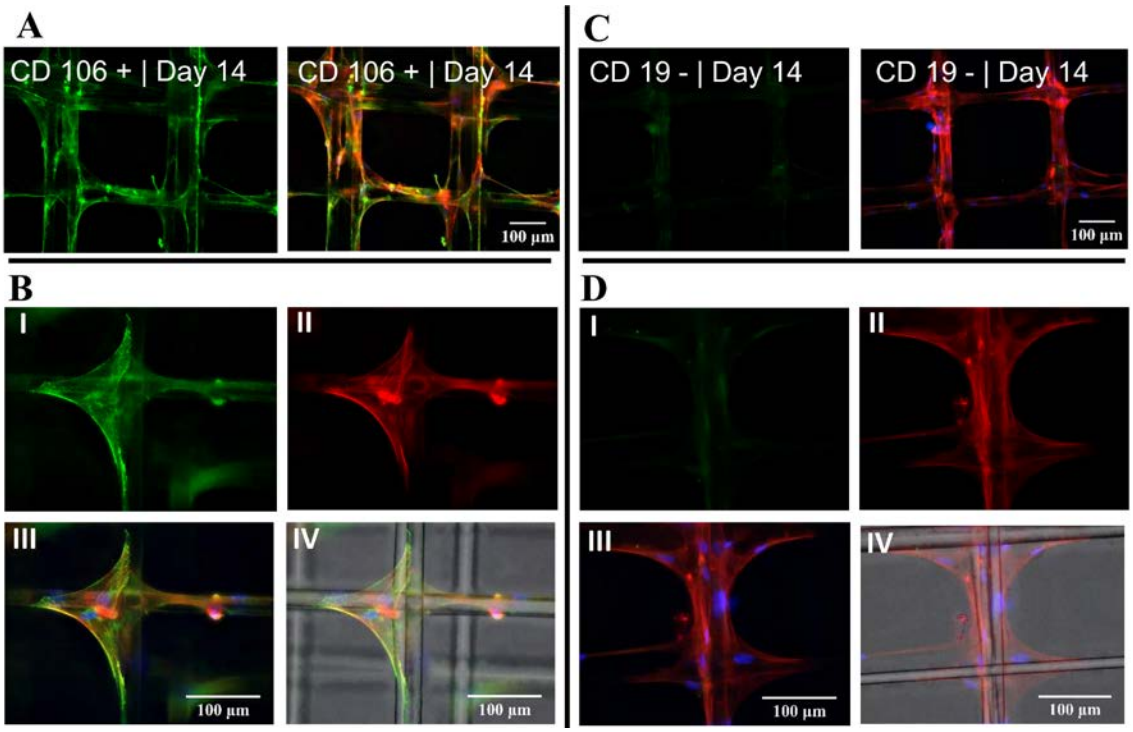

Figure 3. MSCs Positive (CD+) and Negative (CD-) Marker Characterization of MSC s cultured on the MEW|0-90o substrates at Day 14. A) Raw green channel image of CD106+ marker expression (left) and merged fluorescent image (right, red color: actin microfilaments, blue color: nuclei). B) Higher magnification images at the intersection of fibers for CD106+, C) Raw green channel image of CD19- marker expression (left) and merged fluorescent image (right, red color: actin microfilaments, blue color: nuclei), D) Higher magnification images at the intersection of fibers for CD19-. Scale bars: 100 µm.

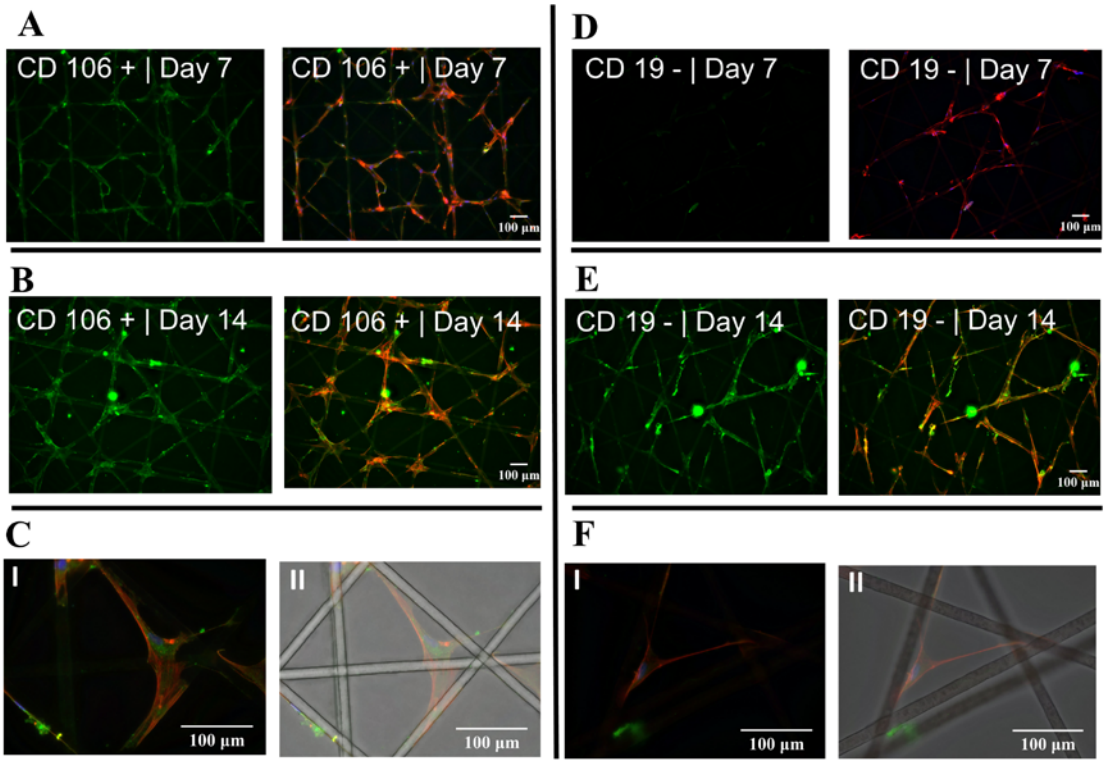

Figure 4. MSCs Positive (CD+) and Negative (CD-) Marker Characterization of MSC s cultured on the MEW|0-45o substrates at Day 7 and 14. A) Raw green channel image of CD106+ marker expression (left) and merged fluorescent image (right, red color: actin microfilaments, blue color: nuclei) at Day 7, B) Raw green channel image of CD106+ marker expression (left) and merged fluorescent image at Day 14, C) Higher magnification images at the intersection of fibers for CD106+, D) Raw green channel image of CD19- marker expression (left) and merged fluorescent image at Day 7, E) Raw green channel image of CD106+ marker expression (left) and merged fluorescent image at Day 14 and F) Higher magnification images at the intersection of fibers for CD19+. Scale bars: 100 µm.

What opportunities for training and professional development has the project provided?

In Year 3, this project has provided support of independent research training and development opportunities for one full-time PhD student. Specifically, the PhD student (Mr. Kai Cao) has led the development of the melt-based electrohydrodynamic printing process for producing scalable 3D biological substrates at the microscale level.

The project has also provided research training exposure and training for one (1) Masters-level student during the academic year along with one (1) summer undergraduate student who both worked on the hydrogel phantom fabrication with the PI during the summer and academic months.

How were the results disseminated to communities of interest?

The results were disseminated through multiple journal publications and media outlets (see PRODUCTS section).

What do you plan to do during the next reporting period to accomplish the goals?

Based on the PI's findings in Years 1-3 of the project, a major task for the next reporting period will be rapid fabrication of spatially heterogeneous biological substrate samples based on an analytically derived general toolpath design model. This will enabled us to embed multiple biological substrate architectures within the same sample for a comparative study of the phenotypic response of mesenchymal stem cells (MSCs) inoculated onto the 3D fabricated substrates. This will enable us to complete the fabrication-characterization cycle, thereby ensuring the printed structural quality and reproducibility required to validate the fundamental manufacturing process work advanced by the proposal. Another significant task for the next reporting period will be to demonstrate in situ electrohydrodynamic printing of the multilayered fibrous graft model directly onto the phantom wound ulcer bed as stipulated in Subtask 2.1 of the proposal. An unexpected challenge that has arisen is the sparking phenomenon associated with the power supply for the electrohydrodynamic printing process in conjunction with the aqueous nature of hydrogel phantom wound bed. In Year 3, although the voltage process parameter has been optimized to mitigate the sparking phenomenon, additional process parameters (including temperature, flowrate, and tip-to-collector distance) will need to be varied in tandem with the high-speed translational stage to enable mechanical stretching with precision fiber placement in both the lateral and layering direction.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Reliably engineering 3D biological templates poses a significant manufacturing challenge. To address this challenge, additive manufacturing (AM) has emerged as a promising approach to attribute unprecedented design complexity to 3D biological systems. Fundamentally, most 3D biological systems in engineered tissue applications are 3D structured biomaterial substrates as cell culture platforms. Current AM methods are able to fabricate biological substrates with complex geometrical features, but not at small scales. Specifically, the limited process resolutions of existing AM methods limits the range of downstream cell functions that can be engineered. Therefore, new manufacturing tools to probe cell-substrate interactions at small scales demands a new manufacturing process design. In this project, the PI advances a high-resolution melt-based electrohydodynamic AM process towards the scalable (fast with small biologically relevant feature sizes) manufacturing of 3D templates for skin grafting. From the standpoint of biological or clinical impact, transplantation of self-renewing stem cells as therapeutic agents is poised to offer new treatment option for recalcitrant ulcer wounds. However, the biological complexity of cells has hampered the translation into the reliable, cost-effective manufacturing of stem cell-based therapies. Although MSCs show great promise for engineered tissues and cell-based regenerative therapies, challenges to clinical adoption of MSC-based products are currently hampered by considerable heterogeneity in the stem cell populations, resulting in significant uncertainty associated with their therapeutic outcomes. The overall premise of this proposed project is accelerated microscale printing of a 3D ordered dermal graft will enable the scalable production of homogeneous MSC populations with a targeted self-renewing phenotype. An innovative strategy based on a novel hybrid electrospinning and 3D printing based additive manufacturing process is proposed herein to fabricate precision-structured three-dimensional geometries as decisive triggers of downstream MSC phenotypes. Furthermore, the novel compression of the time and space scales will be concurrently achieved by identifying the unusual processing conditions and material property ranges that mechanically stretch and align jetted fibers towards fast printing of microscale fibers. Moreover, the ability to quantitatively measure stem cell adhesion as a function of enforced geometrical parameters within a structured material matrix environment promises to open up new avenues of quantitative inquiry into the design of robust preclinical models. Finally, the successful outcome of the proposed research will have significant impact for public health by providing essential insight about the homogeneity of stem cell lineage commitment and their potential for improving therapeutic outcomes. In order to illustrate how fast, small-scale printing can be translated for chronic wound care in the clinical setting, an *in situ* printing-based methodological workflow is proposed to directly fabricate a custom dermal graft onto a phantom excised ulcer wound bed with a contour-matching print process toolpath.

What was the impact on other disciplines?

"Nothing to Report"

What was the impact on technology transfer?

"Nothing to Report"

What was the impact on society beyond science and technology?

"Nothing to Report"

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

"Nothing to Report"

Actual or anticipated problems or delays and actions or plans to resolve them

During the last period of performance, the PI team has made steady progress in testing the Specific Aim 1 hypothesis by identifying the key process and material parameters amenable for uniform mechanical stretching and alignment o the printed fibers. Specifically, in Year 3, the PI team was able to build a new process enclosure in order to address the dual challenges of fluctuating ambient conditions and a sterile printing environment. However, additional unexpected challenges were presented in ensuring sterile print outcomes amenable to a biological time course study along with the pivot from a syringe-based to a pneumatic-based material feed system. The latter has been satisfactorily addressed while the former requires additional modification of the protocol and incorporation of antibiotics with the biological media formulation towards longer duration biological studies on the MSC-seeded precision dermal graft tissue model constructs as articulated in Specific Aim 2. It is expected that an additional requested time of 12 months will enable the PI team satisfactorily address these technical bottlenecks while advancing a methodology that systematically harvests both morphometric and functional MSC data for the precision dermal graft model as articulated in Specific Aim 2.

Changes that had a significant impact on expenditures

"Nothing to Report"

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

"Nothing to Report"

Significant changes in use or care of human subjects

"Nothing to Report"

Significant changes in use or care of vertebrate animals

"Nothing to Report"

Significant changes in use of biohazards and/or select agents

"Nothing to Report"

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

For this reporting period, the PI team has published the following six (6) journal manuscripts supported by this grant:

1. Authors: Kai Cao, Fucheng Zhang, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: Advancing a real-time image-based jet lag tracking methodology for optimizing print parameters and assessing melt electrowritten fiber quality
   Journal: Additive Manufacturing 54, 102764, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

2. Authors: Kai Cao, Fucheng Zhang, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: Quantitative investigation into the design and process parametric effects on the fiber-entrapped residual charge for a polymer melt electrohydrodynamic printing process
   Journal: Macromolecular Materials and Engineering, 2100766, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

3. Authors: Fucheng Zhang, Kai Cao, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: Design, fabrication, and analysis of spatially heterogeneous scaffold by melt electrospinning writing of poly (ε-Caprolactone)
   Journal: Journal of Applied Polymer Science, 52235, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

4. Authors: Fucheng Zhang, Kai Cao, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: Effects of printing sequence on the printing accuracy of melt electrowriting scaffolds
   Journal: Macromolecular Materials and Engineering, 2200222, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

5. Authors: Ahmadreza Zaeri, Kai Cao, Fucheng Zhang, Ralf Zgeib, Robert C. Chang
   Title: A review of the structural and physical properties that govern cell interactions with structured biomaterials enabled by additive manufacturing
   Journal: Bioprinting, e00201, 2002
   Status of Publication: Published
   Acknowledgement of federal support: Yes

6. Authors: Ahmadreza Zaeri, Ralf Zgeib, Kai Cao, Fucheng Zhang, Robert C. Chang
   Title: Numerical analysis on the effects of microfluidic-based bioprinting parameters on the microfiber geometrical outcomes
   Journal: Scientific Reports 12 (1), 1-16, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications.

"Nothing to Report"

Other publications, conference papers and presentations.

"Nothing to Report"

- **Website(s) or other Internet site(s)**

"Nothing to Report"

- **Technologies or techniques**

"Nothing to Report"

- **Inventions, patent applications, and/or licenses**

"Nothing to Report"

- **Other Products**

"Nothing to Report"

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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1. Name: Kai Cao
   Project Role: Graduate Student
   Researcher Identifier (e.g. ORCID ID): N/A
   Nearest person month worked: 12
   Contribution to Project: bioprinting of phantom wound ulcer bed; MSC phenotype characterization; melt-based electrohydrodynamic printing process optimization; design and build of variable temperature collector module; ongoing parametric studies for microscale layered 3D templates
   Funding Support: Internal funding & DOD Award # W81XWH-19-1-0158

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report"

What other organizations were involved as partners?

"Nothing to Report"

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS:
   QUAD CHARTS:

9. **APPENDICES:**