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

PRINCIPAL INVESTIGATOR: Robert C. Chang

CONTRACTING ORGANIZATION: Stevens Institute of Technology, Hoboken, NJ

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Hoboken, NJ 07030

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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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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 (100% 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 (100% completion) at 18 months: Production of a homogeneous self-renewing MSC population as a dermal graft model.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Activities:

In Year 4 of the project, the PI team updated the bioprinting algorithm and software for the phantom wound ulcer bed along completion of the investigation into MSC phenotype characterization on the heterogeneous 3D structured biomaterial substrates.

A. Software for Bioprinting of phantom wound ulcer bed

Based on the previously mentioned algorithm and workflow, a fully customized software with multiple functions for bioprinter control is presented, which includes 3 major modules: video monitoring, tool-path generator, and motion controller, as shown in Fig. 6. These 3 major modules are developed as a robotic-assisted bioprinting system. The first module, the video monitoring module, consists of the ArUco marker detection function and homography transformation method between 3D objects and the printing bed. The second module, the tool-path generator module, includes 2D tool-path generation and 3D non-planar tool-path generation. The 2D toolpath generation that only covers XY dimensions uses a continuous tool-path generation method based on results from Year 3 of the project, which serves as a basis for 3D tool-path generation. The 2D tool-path generation is based on concave polygon triangulation algorithms, which could determine whether a point is within a triangle shape or not and then create a zig-zag tool-path to fill the region in order. Based on the developed 2D tool-path generation, the 3D non-planar tool-path generation is built upon the grid map and shape match algorithms for 2D contour registration between a 2D image and the 2D projection of 3D *.STL model. The 3D non-planar tool-path generation is the main contribution of this paper. The third module is the motion controller that controls the motor movement and extruder of the printer using G-code and stepper motor controller.

The software is presented as a bioprinting controller with a graphic user interface (GUI) (as shown in Fig. 7), which contains 3D printer manual calibration, configuration and control panel, imaging preview and processing window, *.STL file preview, printing path generation and visualization window. This GUI enables interaction between the user and the 3D printer including 3D printer operation and in situ bioprinting via non-planar tool-path planning. Specifically, this software has a real-time live window showing the bioprinting platform with 3D objects along with the extracted wound region of interest (ROI) for bioprinting (red area in Fig. 1). Starting with manual positioning of the needle within the interactive imaging previewing window, the bioprinter could automatically calculate the needles and object position within the image. Subsequently, the software automatically calculates the object size, orientation, ROI boundary, size, orientation, and the 3D path for the printed by clicking the corresponding buttons. With this one-click solution, a fully automated bioprinting system enabling in situ printing onto a non-planar 3D object becomes a potentially practical solution for skin burn surgical interventions.

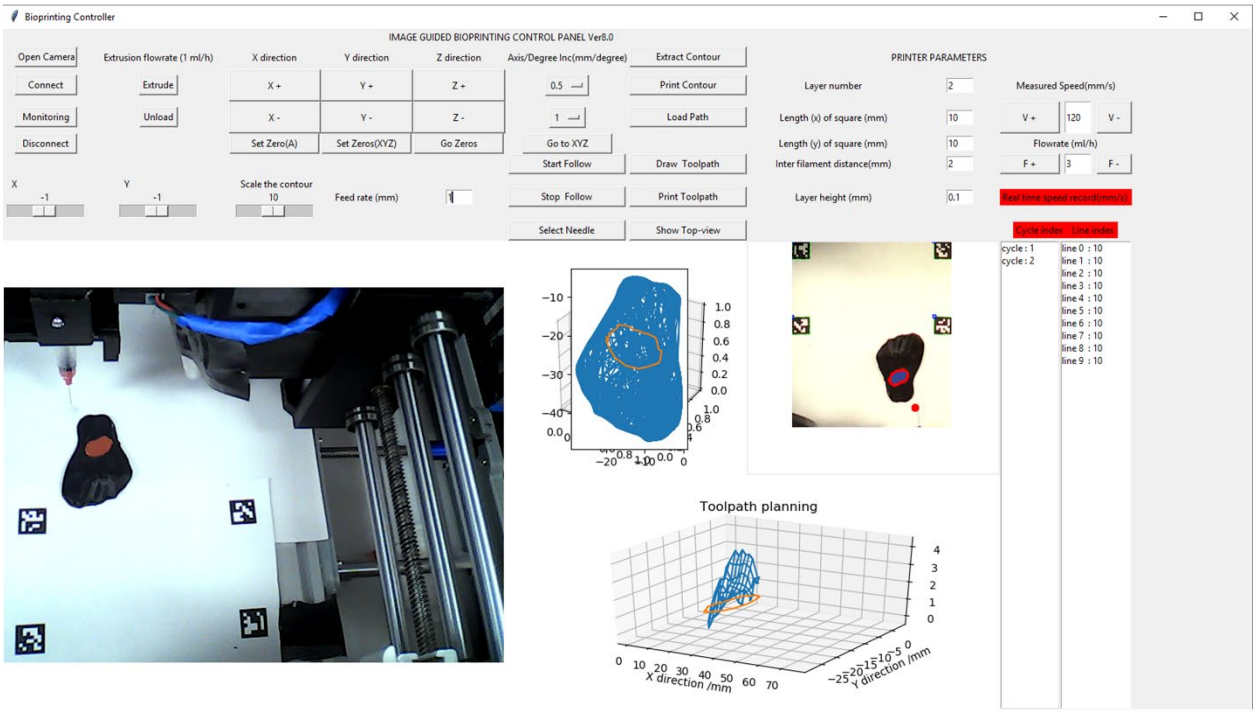

Figure 1. Customized software GUI based on Python(tk) for in situ 3D non-planar bioprinting.

B. MSC Phenotype Characterization on Heterogeneous Scaffolds

In Year 45, 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|0-90o substrate at the prescribed time points. It is observed that MSCs maintain their stemness during the first week of culture on MEW|0-45o substrates, while they lose their MSC phenotype sometime during the second week of culture. In Year 6, heterogeneous scaffolds of varying porous architecture (e.g. 0-45 and 0-90) are fabricated to evaluate the cell fate or differentiation outcomes for the MSCs seeded on the heterogeneous scaffolds compared to a 2D monolayer control substrate. For the 2D monolayer control substrate (Figure 2), it is found that the MSCs lose their stemness wherein, at 33 day culture time course, a mixed population of fat, bone, and cartilage cells is confirmed based on FABP4, osteoclacin, and aggregan fluorescent staining, respectively. In contrast, upon losing stemness at approximately one week, MSCs seeded on 0-45 substrate architectures (Figure 3) are determined to all become fat cells. Based on this finding, it can be concluded that the 0-45 substrate artchitecture can yield a homogeneous, monolithic population of differentiated cells.

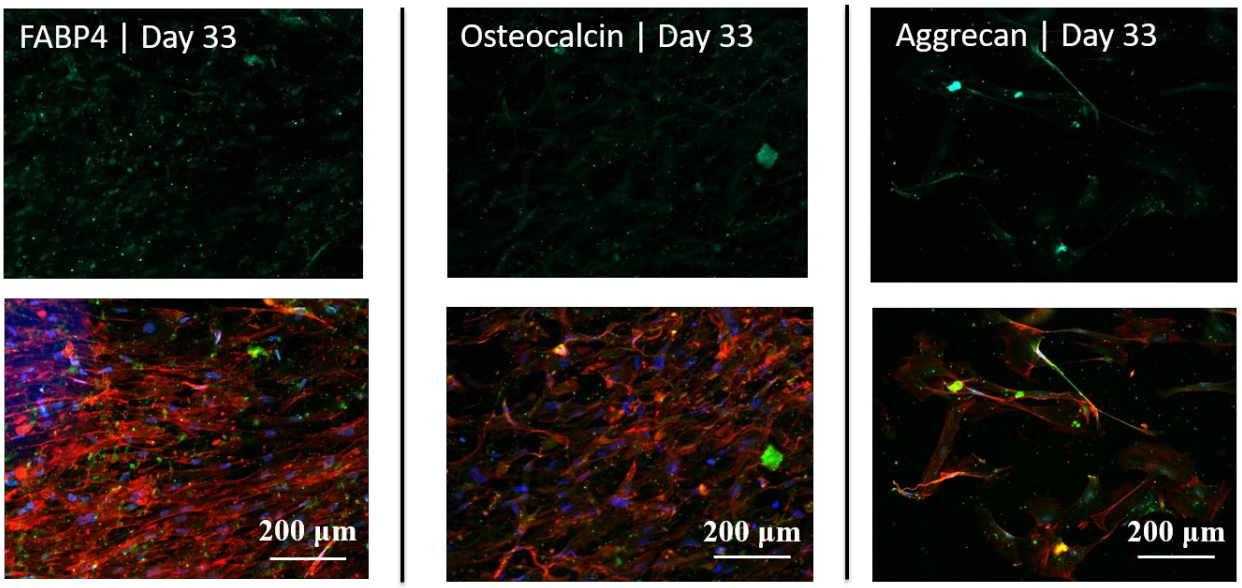

Figure 2. Fluorescent staining images for MSCs seeded on conventional 2D monolayer substrates with terminal tri-lineage differentiation at Day 33 for fat, bone, and cartilage based on positive fluorescent staining of FABP4, osteocalcin, and aggrecan, respectively.

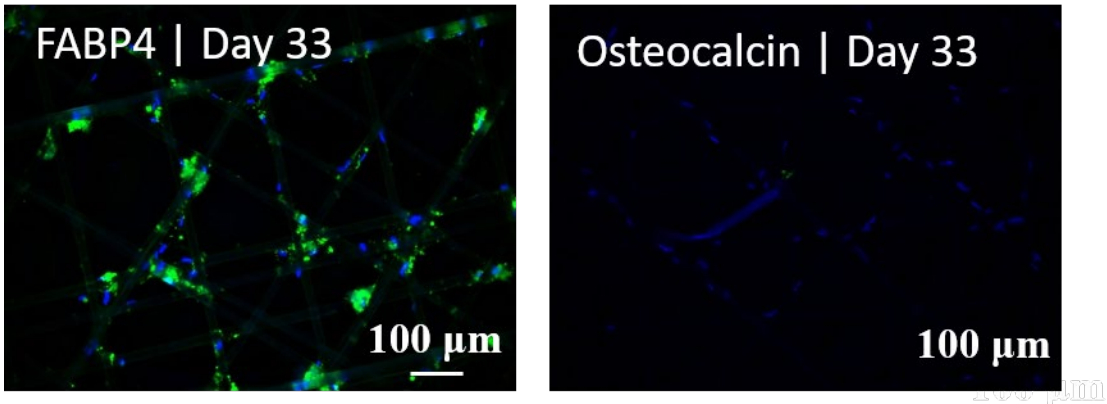

Figure 3. MSCs Positive (CD+) and Negative (CD-) Marker Characterization of MSC s cultured on the MEW|0-90o substrates at Day 14. A) Raw green channel image of CD106+ marker expression (left) and merged fluorescent image (right, red color: actin microfilaments, blue color: nuclei). B) Higher magnification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

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

In Year 4, this project has provided support of independent research training and development opportunities for one full-time PhD student. Specifically, the PhD student (Mr. Fucheng Zhang) 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

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

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

"Nothing to Report"

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

"Nothing to Report"

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

"Nothing to Report"

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

"Nothing to Report"

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

"Nothing to Report"

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

"Nothing to Report"

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

"Nothing to Report"

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

"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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

For this reporting period, the PI team has published the following five (5) journal manuscripts supported by this grant:

1. Authors: Kai Cao, Fucheng Zhang, Bijun Wang, Yuning Sun, Ahmadreza Zaeri, Ralf Zgeib, Mo Mansouri, Robert C. Chang
   Title: Analytical interpretation of microscale fiber deviation in designing for polymer melt electrohydrodynamic-based additive manufacturing
   Journal: Additive Manufacturing 58, 103035, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

2. Authors: Kai Cao, Fucheng Zhang, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: A holistic model for melt electrowritten three-dimensional structured materials based on residual charge
   Journal: International Journal of Bioprinting 9(2), 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

3. Authors: Kai Cao, Fucheng Zhang, Ahmadreza Zaeri, Ralf Zgeib, Mellina Calzolaio, Robert C. Chang
   Title: Advances in design and quality of melt electrowritten scaffolds
   Journal: Materials & Design 111618, 2023
   Status of Publication: Published
   Acknowledgement of federal support: Yes

4. Authors: Fucheng Zhang, Kai Cao, Ahmadreza Zaeri, Ralf Zgeib, Christian Buckley, Robert C. Chang
   Title: Design, fabrication, and characterization of tubular scaffolds by way of a melt electrowriting process
   Journal: Additive Manufacturing 62, 103383, 2023
   Status of Publication: Published
   Acknowledgement of federal support: Yes

5. Authors: Fucheng Zhang, Kai Cao, Ahmadreza Zaeri, Ralf Zgeib, Robert C. Chang
   Title: Effects of scaffold design parameters on the printing accuracy for melt electrowriting
   Journal: Journal of Manufacturing Processes 81, 177-190, 2022
   Status of Publication: Published
   Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

"Nothing to Report"

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

"Nothing to Report"

Website(s) or other Internet site(s)
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

"Nothing to Report"

Technologies or techniques
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

"Nothing to Report"

Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

"Nothing to Report"

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

"Nothing to Report"

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

1. Name: Fucheng Zhang
   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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

"Nothing to Report"

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

"Nothing to Report"

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.