TASK ORDER NUMBER: W81XWH-15-9-0001

MTEC RESEARCH PROJECT NUMBER: MTEC-18-05-PeripheralNerve-0028

EGS NUMBER: MT180011

TITLE: Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System

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CONTRACTING ORGANIZATION: Medical Technology Enterprise Consortium (MTEC)

REPORT DATE: 10/25/2020

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
10/25/2020	Annual Report	10/01/2019-09/30/2020

4. TITLE AND SUBTITLE
Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neutrophic Factor Release System

5a. CONTRACT NUMBER
W81XWH-15-9-0001

5b. GRANT NUMBER
N/A

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER
MT180011

5e. TASK NUMBER
W81XWH-18-9-0009

5f. WORK UNIT NUMBER
N/A

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wake Forest University
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8. PERFORMING ORGANIZATION REPORT

2017-614-002

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
N/A

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		

Standard Form 298 (Rev. 8-98)

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Annual Technical Status Report for

Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System Research

Research Project No. 2017-614-002

EGS# MT180011

Reporting Period: 10/01/2019 – 09/30/2020

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1. Project Status

a. Accomplishments

i. Milestone completion

1. Task 1.1/D1.1 Determine the effects of neurotrophic factors on skeletal muscle development and acetylcholine receptor (AChR) cluster pre-formation on the bioprinted muscle construct (35% completion during this reporting period, 80% total completion)
    a. Establishing the optimal conditions of agrin treatment to efficiently induce AChR cluster pre-formation on human muscle progenitor cells (hMPCs) in the bioprinted muscle construct
    b. Optimizing the bioink composition for 3D bioprinting of muscle constructs with hMPCs
    c. Determining the viability and differentiation efficiency of hMPCs with agrin treatments in bioprinted constructs
    d. Establishing the effect of neurotrophic factors (ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF)) on the hMPC viability and proliferation in the bioprinted muscle construct
    e. Optimizing the protocol to fabricate PLGA (poly-lactic-co-glycolic-acid) microspheres as a vehicle for controlled release of neurotrophic factors in the bioprinted muscle constructs
    f. Evaluating the kinetics of neurotrophic factor release from PLGA microspheres in the bioprinted muscle construct

2. Task 1.2/D1.2 Demonstrate neuromuscular junction (NMJ) formation and innervation on the bioprinted skeletal muscle constructs, *in vitro* (30% completion during this reporting period, 80% total completion)
    a. Establishing the synergistic effect of CNTF and GDNF on directed growth of neurites using embryonic chicken dorsal root ganglia (DRG) in a 2D chemotaxis assay. Optimizing the quantitative parameters for the induction of directional growth of neurites.
    b. Demonstrating the ability of CNTF and GDNF to induce neurite ingrowth into 3D bioprinted constructs
    c. Optimizing the protocol to incorporate PLGA microspheres into bioprinted skeletal muscle constructs
    d. Demonstrating NMJ formation between the agrin-induced AChR clusters and DRG neurites in the presence of CNTF and GDNF

3. Task 1.3/D1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model (42% completion during this reporting period, 47% total completion)
    a. Carrying out preliminary tests of the surgical procedures and personnel training using rat cadavers (model creation)

b. Carrying out a pilot *in vivo* study - implantation of AChR cluster pre-formed and neurotrophic factors-incorporated bioprinted muscle constructs in the ectopic transposed nerve model in rats

ii. Published papers

1. Ji Hyun Kim et al. Neural cell integration into 3d bioprinted skeletal muscle constructs accelerates restoration of muscle function. Nature Communications 11, 1025, 3644 (2020)
2. Vladimir Mashanov et al. Synergistic effect of CNTF and GDNF on directed neurite growth in chick embryo dorsal root ganglia. PLoS ONE 15(10): e0240235 (2020).
3. Hyeongjin Lee et al. Effect of hierarchical scaffold consisting of aligned dECM nanofibers and poly(lactide-co-glycolide) struts on the orientation and maturation of human muscle progenitor cells. ACS Appl. Mater. Interfaces, 11, 39449-39458 (2019)

b. Reportable Outcomes

i. Parameters and conditions for reliable induction of AChR cluster formation by agrin treatment in differentiated human muscle cells of the bioprinted muscle construct were established (8 days of agrin treatment at 150 ng/mL).

ii. Bioink parameters for bioprinting skeletal muscle constructs were tested and optimized. Fibrin concentration at 20 mg/mL was established in terms of hMPC differentiation to form muscle fibers within the bioprinted construct.

iii. The viability and differentiation efficiency of AChR cluster pre-formed bioprinted muscle construct were determined at the optimized bioink composition (> 70% cell viability, 55 – 68% of differentiation efficiency). Agrin treatment for AChR cluster pre-formation did not affect the viability and differentiation efficiency of the bioprinted muscle construct.

iv. Comprehensive statistical analysis showed that the combination of neurotrophic factors, CNTF and GDNF (10 ng/mL each), induced directed neurite growth.

v. The synergistic effect of the CNTF and GDNF on directional neurite growth was demonstrated in various concentrations.

vi. The combination of the neurotrophic factors - CNTF and GDNF - chosen to induce directional neurite growth had no deleterious effect on hMPCs viability and proliferation.

vii. Incorporation of CNTF and GDNF into the bioink induced the neurite growth into the bioprinted constructs.

viii. A protocol to fabricate PLGA microspheres was developed and optimized.

ix. Neurotrophic factors (CNTF and GDNF) were released from the PLGA microspheres in a sustained manner. The PLGA microspheres can be used as carriers for the controlled and sustained release of the neurotrophic factors in the bioprinted muscle construct.

x. Optimal bioprinting parameters for incorporating PLGA microspheres into bioprinted skeletal muscle constructs were determined in terms of printability and cell viability. The incorporated microspheres with a concentration of 5 mg/ml did not affect cell viability. The microsphere-incorporated bioprinted muscle constructs showed a high cell viability, 87.8%.

xi. In *in vitro* co-culture experiments, patient-derived hMPCs with pre-formed AChR clusters could form NMJs with neurite outgrowth from the DRG in the presence of the neurotrophic factors, CNTF and GDNF.

xii. The ectopic transposed nerve implantation model was successfully established. The reliability of the animal model was confirmed. The research team was trained on the procedures for consistency.

xiii. The research team has practiced tissue sample evaluation techniques associated with electromyography (EMG), specimen preparation, tissue sectioning, histology, immunocytochemistry, and imaging. In addition, the research team has prepared cells, animals, materials, and supplies required for the *in vivo* studies.

xiv. A pilot *in vivo* study was initiated using the ectopic transposed nerve implantation model in rats.

c. Progress Detail

(1) Optimizing AChR cluster pre-formation on differentiated human muscle cells with agrin treatment in 3D bioprinted skeletal muscle constructs

Pre-formation of AChR clusters on differentiated muscle cells in the bioengineered skeletal muscle construct can facilitate rapid innervation of the implanted muscle by the host's nervous system. Accelerated innervation will improve the viability, longevity, and functionality of the implanted bioprinted muscle constructs *in vivo*. In our previous pilot experiments, we demonstrated that agrin treatment (R&D Systems, 6624-AG), added to the differentiation medium (DM) at 150 ng/mL, pre-formed AChR clusters on differentiated myotubes after 5-8 days in 2D culture.

During this reporting period, we proceeded to test whether this treatment will also facilitate the AChR cluster development in 3D printed constructs containing the differentiated human muscle cells. The bioprinted constructs cultured in the DM were subjected to one of the following three treatments:

- Group 1. Without agrin for 8 days (control group) (n = 42)
- Group 2: Without agrin for 3 days and then with agrin (150 ng/mL) for 5 days (3d DM + 5d agrin) (n = 26)
- Group 3: With agrin (150 ng/mL) for 8 days (8d agrin) (n = 33)

The samples were then fixed and processed for immunofluorescent staining with anti-myosin heavy chain (MHC) and anti-AChR antibodies **(Fig. 1)**.

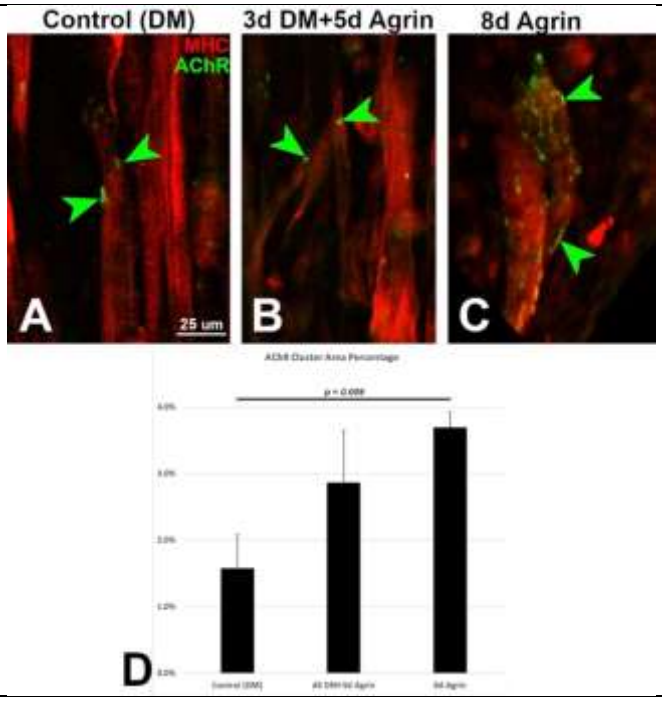


Figure 1. Effect of two different agrin treatment regimens (3 days DM followed by 5 days of agrin at 150 ng/ml and 8 days of continuous agrin treatment at the same concentration) on the formation of AChR clusters in differentiating hMPCs in the bioprinted constructs. (A - C) Representative micrographs of immunostained constructs of the control cohort and the two cohorts subjected to the respective agrin treatment. The samples were immunostained with antibodies against the human myosin heavy chain (MHC, red) (DSHB, MF20) and acetylcholine receptor (AChR, green) (Sigma, M217). AChR clusters are indicated by arrowheads. (D) Continuous agrin treatment for 8 days at 150 ng/mL results in a significant increase of the AChR cluster area percentage in differentiating hMPCs compared to the control cohort. N = 42 cells for control (DM), 26 cells for 3d DM + 5d agrin, and 33 cells for 8d agrin.

The constructs that were continuously treated with agrin for 8 days resulted in a significant increase in the area of AChR clusters (%) in differentiated muscle cells compared to the control group. This group showed a significant increase (~2.3 fold) in the relative area of AChR clusters in differentiated muscle cells (Fig. 1). Although there were no significant differences between the 3d DM+5d agrin and 8d agrin group in the area of AChR clusters, the 8d agrin group showed a higher mean value than the 3d DM+5d agrin group.

These results indicate that agrin treatment can increase AChR cluster pre-formation on the printed hMPCs. Therefore, the regimen of 8d Agrin treatment was chosen to prepare the constructs for the *in vivo* implantation study.

(2) Optimizing the bioink composition

We have previously developed a methodology to generate viable human muscle constructs (10 mm × 10 mm × 3 mm) using hMPCs and the 3D Integrated Tissue-Organ Printing (ITOP) system. During this reporting period, we further optimized the composition of the bioink. We determined the optimal concentration of fibrinogen in the bioink to enhance the differentiation of hMPCs. Only the hMPCs at a concentration of 20 mg/mL of fibrinogen in the constructs started expressing MHC, a hallmark of differentiation in this cell type, after 7 days in culture in the differentiation medium (Fig. 2). hMPCs with higher concentrations of fibrinogen (30 mg/mL and 40 mg/mL) did not show MHC expressions.

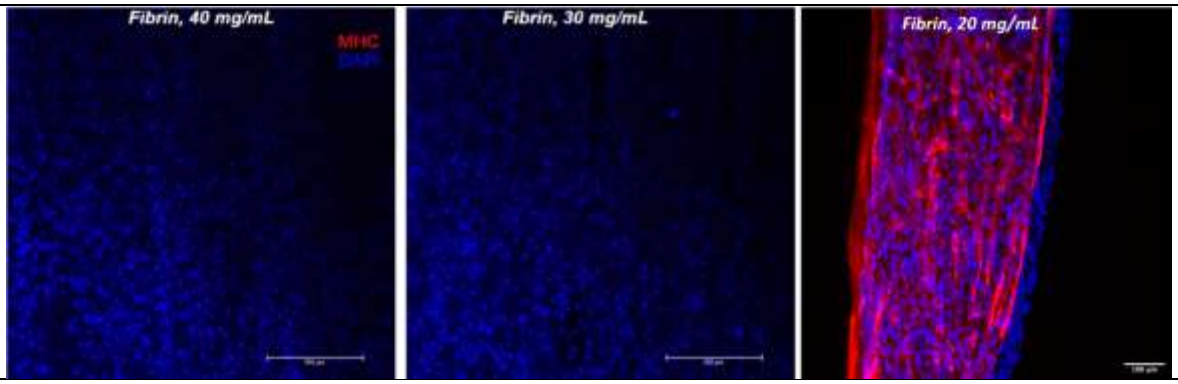


Figure 2. Effect of fibrin concentration in the bioink on hMPCs differentiation in bioprinted muscle constructs. The constructs were cultured in the differentiation medium for 7 days and then immunostained with antibodies against the MHC. The nuclei were stained with DAPI. Only cells in the constructs with 20 mg/mL of fibrinogen show proper differentiation.

(3) Determining the viability and differentiation efficiency of hMPCs in bioprinted constructs

After having optimized the bioprinting parameters and the composition of the bioink, we quantitatively determined the viability and differentiation efficiency of hMPCs in the bioprnted muscle constructs.. The purpose of this study is to pre-form AChR clusters on differentiated muscle cells in bioprinted muscle construct before implantation for accelerating innervation *in vivo*. AChR clusters can be pre-formed on the differentiated muscle fibers, not on the undifferentiated hMPCs. In line with this, *in vitro* incubation of the bioprinted muscle construct before implantation is necessary to achieve proper myogenic differentiation of hMPCs and AChR clusters pre-formation on the differentiated muscle cells. Therefore, it is crucial to determine the viability and differentiation efficiency of the bioprinted muscle constructs after *in vitro* culture with agrin for 8 days before implantation.

For the tests, the bioprinted constructs were fabricated using hMPCs and were separated into three cohorts;

- Group 1. DM without agrin for 8 days (Control, d8)
- Group 2. DM with agrin (150 ng/mL) for 8 days (Agrin, d8)
- Group 3. DM without agrin for 3 days and then DM with agrin (150 ng/mL) for 5 days (3D DM + 5D Agrin)

(a) Cell viability

Cell viability in the bioprinted muscle constructs was determined with the Live/Dead Assay (Molecular Probes). The stained constructs were imaged using a confocal microscope, and the number of live cells (green) and dead cells (red) were counted using the Fiji/ImageJ software. The cell viability in each experimental group was calculated as the percentage of live cells (green) relative to the total number of cells (live cells + dead cells (red)) (%).

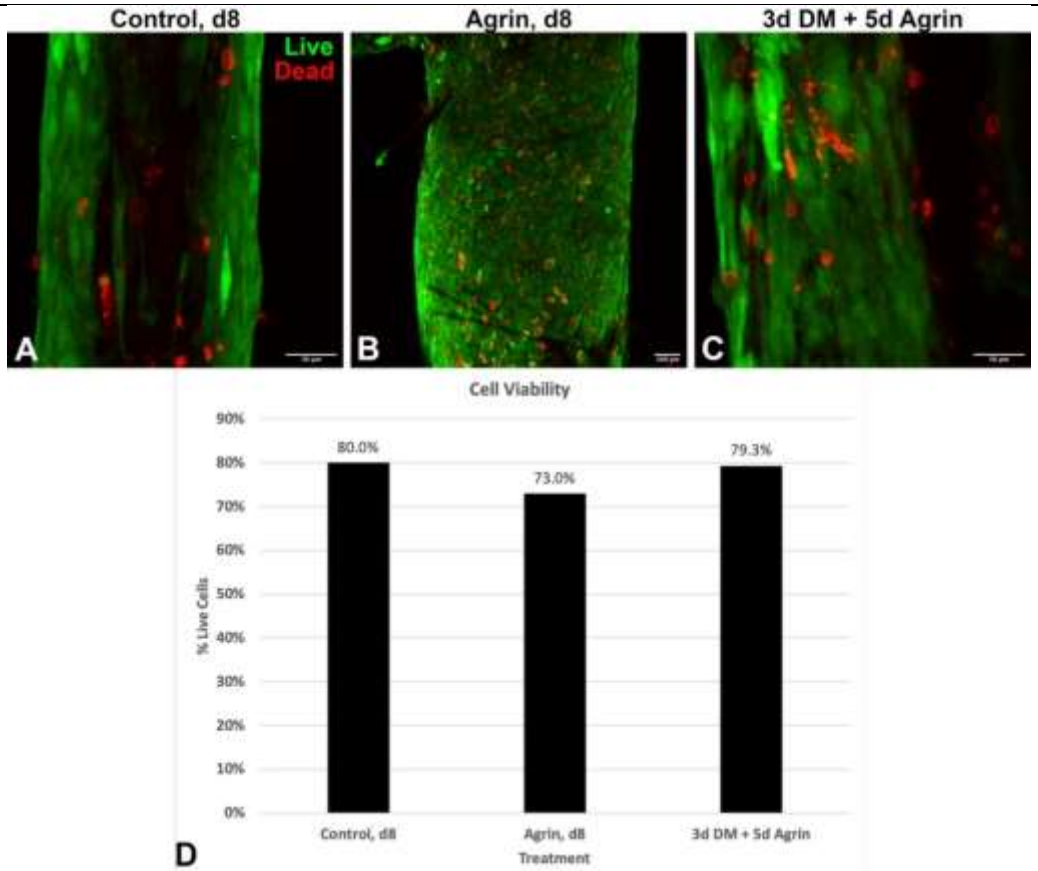


Figure 3. Cell viability in 3D printed constructs determined with the Live/Dead Assay (Molecular Probes) on day 8 of *in vitro* culture. (A—C) Representative micrographs. Image stacks were taken with a confocal microscope, then were used to make Z-projections with Fiji/ImageJ. Live cells are shown in green, and nuclei of dead cells are shown in red. (D) Live cell percentage in the constructs. Three different treatments were analyzed: the control cohort incubated in pure differentiation medium (DM) without agrin; the second cohort treated with agrin (150 ng/mL) contained DM to facilitate the formation of AChR clusters; the constructs of the third cohort were first cultured in DM for 3 days and then subjected to agrin treatment at the same concentration. All three treatment groups show high cell viability (>70%).

This analysis showed the robust viability of the hMPCs (73—80%) in all three treatment groups after 8 days of *in vitro* culture (Fig. 3). Therefore, the addition of agrin to culture medium with the purpose of induction of AChR cluster formation did not affect the viability of the printed hMPCs in the bioprinted muscle constructs (compare Control vs. Agrin 8d and 3d DM + 5d Agrin).

(b) Differentiation efficiency

The differentiation efficiency of hMPCs in the bioprinted constructs was investigated using immunofluorescence staining for MHC. The percentage of nuclei in MHC-positive cells relative to the total number of nuclei in the sample was used as a metric of muscle differentiation. It reflects the proportion of the cells that fused into syncytial myotubes that, upon differentiation, start accumulating MHC in their cytoplasm.

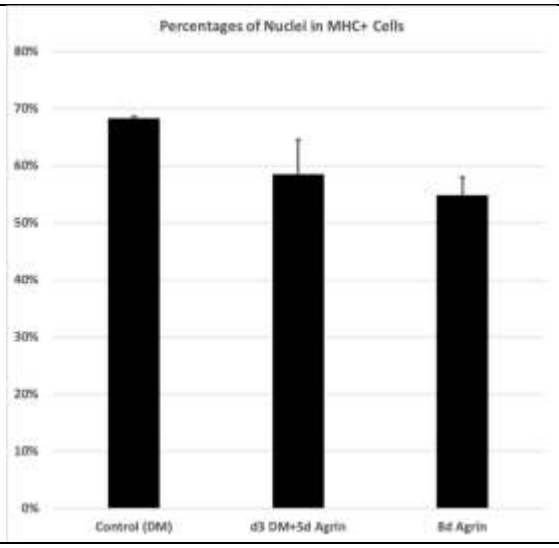


Figure 4. Percentage of nuclei in MHC-positive cells (i.e., differentiating skeletal muscle cells) relative to the total number of nuclei in the sample. After bioprinting, the constructs were subjected to one of the following treatments: the control cohort was incubated in pure differentiation medium (DM) without agrin; the second cohort was treated with agrin (150 ng/mL) contained DM to facilitate the formation of AChR clusters; the constructs of the third cohort were first cultured in DM for 3 days and then subjected to agrin treatment at the same concentration. Muscle progenitor cells in all treatment groups show robust differentiation. N = 3 - 4 per group.

Muscle progenitor cells in all three treatment cohorts showed robust differentiation (55 – 68% of progenitor cells would fuse to form muscle fibers) with no statistically significant variation between the groups (n = 3 - 4 per group, one-way ANOVA F(2, 7) = 0.199) **(Fig. 4)**. The results indicate that hMPCs can be efficiently differentiated into muscle fibers in bioprinted constructs. Agrin treatment to induce the AChR cluster formation did not interfere with the normal progression of muscle differentiation in the bioprinted muscle construct.

(4) Directed neurite outgrowth by the combination of CNTF and GDNF

Earlier, we demonstrated that CNTF and GDNF act synergistically to facilitate directional neurite outgrowth from embryonic chicken DRGs. Initially, we used a single metric – forward migration index – to quantify the neurite growth. During this reporting period, we continued with the statistical analysis of the data, and calculated other metrics commonly used in the field to corroborate the initial analysis further. These results were recently accepted for publication by a peer-reviewed scientific journal (Mashanov et al., PLoS ONE, 2020; Please see the "**ii. Published papers**" on Page 6).

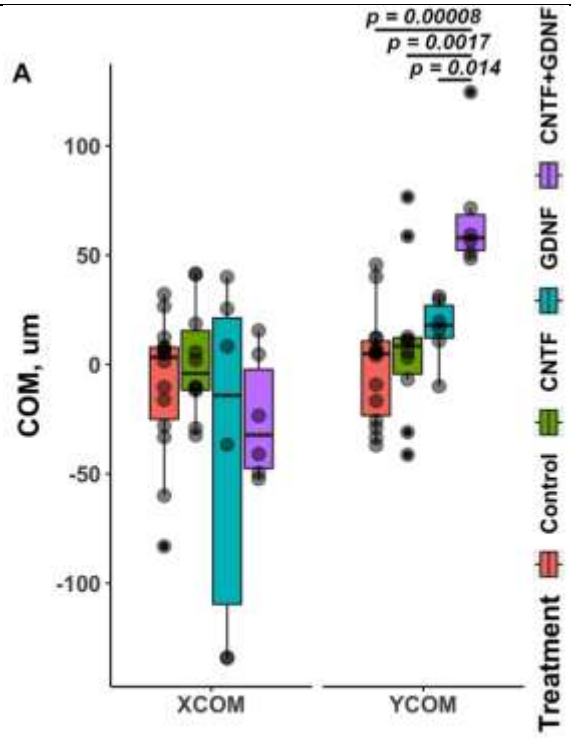
To quantify the directed neurite outgrowth (i.e., to demonstrate the preferential growth towards the source of the neurotrophic factors in our *in vitro* guidance assay), we used two additional metrics:

(a) Center of mass (COM) displacement
(b) Rayleigh test

(a) COM displacement

The COM was calculated as the geometrical average of the coordinates corresponding to the distal ends of all neurites growing from a given ganglion. COM displacement from the coordinate plane center represents the direction in which the neurites preferentially grow. In the case of the directed growth, COM is expected to shift in the positive direction along the y-axis (YCOM), which is aligned parallel to the gradient of the neurotrophic factors, with no significant displacement along the x-axis (XCOM), which is oriented perpendicular to the gradient.

In the first experiment, the DRG explants were treated with CNTF (10 ng/mL) and GDNF (10 ng/mL), either individually or in combination. No significant variation in the COM displacement along the x-axis was observed across the treatment groups (one-way ANOVA F(3, 32) = 1.357, p = 0.247) **(Fig. 5A)**. The DRGs treated with the combination of CNTF and GDNF showed a significant displacement in the positive direction along the y-axis towards the source of the neurotrophic factors that were significantly higher than that in the control group ($p = 8.2 \times 10^{-5}$) and in the samples treated individually with either CNTF ($p = 1.7 \times 10^{-3}$) or GDNF ($p = 1.4 \times 10^{-2}$). Neither of the latter two cohorts treated with the neurotrophic factors individually was statistically different from the control group **(Fig. 5A)**.



B

Treatment	Rayleigh Test Average P-value
Control	0.130
CNTF	0.357
GDNF	0.071
CNTF+GDNF	0.033

Figure 5. Quantitative effect of CNTF and GDNF on directed growth of DRG neurites when applied individually or in combination at 10 ng/mL each (48 hours in culture). **(A)** Center of mass (COM) displacement in µm along the x-axis (XCOM) and y-axis (YCOM). **(B)** Average p-value of Rayleigh test for each treatment group.

(b) Rayleigh test

Another metric used to assess directed neurite growth was the Rayleigh test, which determines whether the coordinates of the growing neurites' distal ends follow the uniform angular distribution. In the case of the directed growth, the null hypothesis of uniformity is rejected, with the p-value of the test being below 0.05. After 48 hours in culture, only the combination of CNTF and GDNF had the p-value of the Rayleigh test lower than 0.05 (p = 0.033). The p-value in the control cohort, and the groups treated separately with CNTF and GDNF, were above the threshold level of 0.05 **(Fig. 5B)**.

Thus, all three metrics that we used to assess the directed growth – forward migration index (reported earlier), the COM displacement, and Rayleigh test (reported here) - corroborated each other, showing a

synergistic effect of CNTF and GDNF on directional neurite growth. In contrast, when applied individually, these neurotrophic factors lose the ability to induce directed growth.

Additionally, we analyzed other growth parameters that do not directly measure growth in a specific direction but may still be affected by the neurotrophic factors. These parameters included:

(a) directness
(b) longest neurite length
(c) average neurite length
(d) number of neurites growing out from a given DRG

Directness is a measure of how close trajectories of growing neurites are to the straight line. It was calculated as a ratio of the Euclidean distance between the neurite's proximal and distal ends to the neurite's total length. A directness of "1" indicates that neurites grow along straight-line trajectories. Our data suggest that the use of CNTF and GDNF as chemotaxis cues, either in combination or individually at 10 ng/mL each, did not result in any significant changes in the shape of neurite trajectories (one-way ANOVA $F(3, 32) = 1.942$, $p = 0.143$) **(Fig. 6A)**.

As to the other metrics, the combination of CNTF and GDNF (10 ng/mL each) did not cause any changes in the number of neurites growing out from DRGs, nor did it affect the maximum length of neurites. However, this combined treatment significantly ($p = 0.012$) increased the average length of neurites by a factor of ~1.5 compared to the control group cultured in the absence of neurotrophic factor gradient **(Fig. 6B–D)**. Interestingly, GDNF alone at 10 ng/mL caused a significant increase in all three parameters (the maximum neurite length, average neurite length, and the number of neurites per DRG) compared to the control group **(Fig. 6B–D)**.

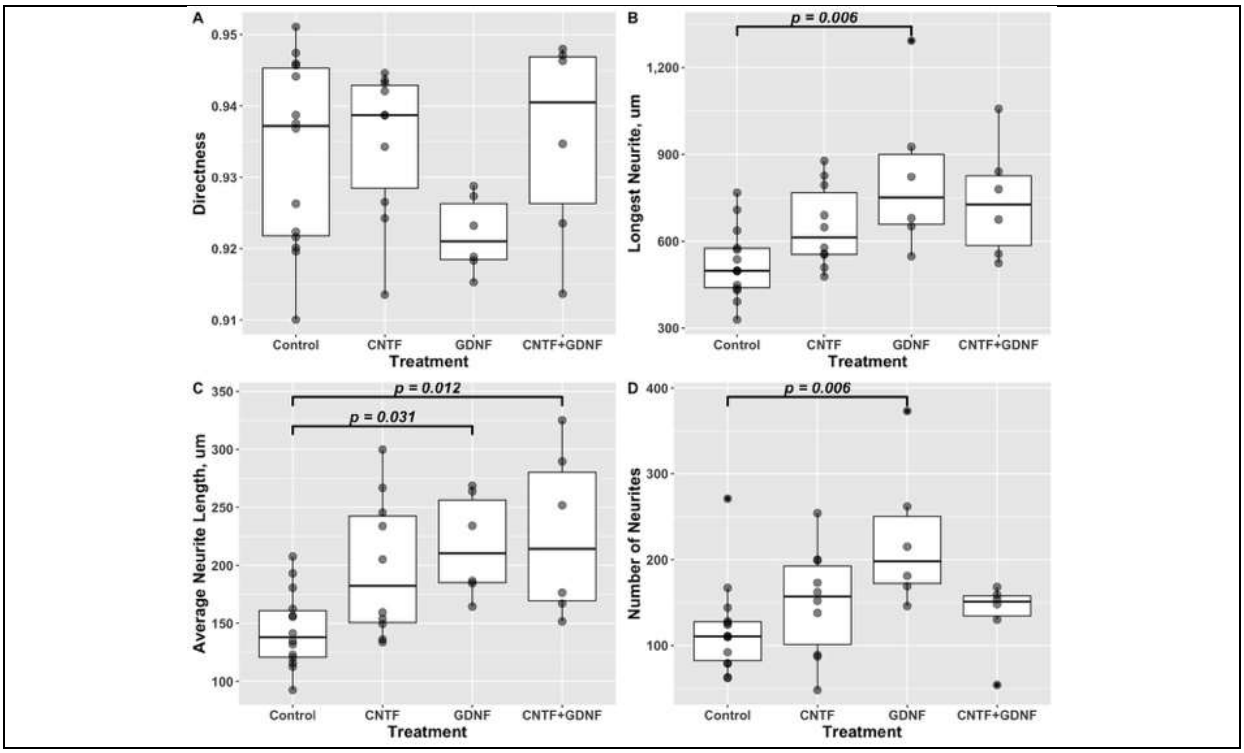


Figure 6. Quantitative effect of CNTF and GDNF on growth properties of DRG neurites when applied individually or in combination at 10 ng/mL each (48 hours in culture). **(A)** Directness. No significant variation was detected among the treatment groups (One-way ANOVA $F(3, 32) = 1.942$, $p = 0.143$). **(B)** Length of the longest neurite in DRGs. There was a significant variation among the treatment groups (One-way ANOVA $F(3, 32) = 5.137$, $p = 5.17\times10^{-3}$). Tukey post-hoc test revealed that the length of the longest neurite significantly increased in the cohort treated with GDNF compared to the control group. **(C)** Average neurite length. One-way ANOVA detected significant changes among the groups in response to the gradient of neurotrophic factors ($F(3, 32) = 5.318$, $p = 4.35 \times 10^{-3}$). The cohorts exposed to GDNF and the combination of CNTF and GDNF showed a significant increase in the mean neurite length compared to the control group. **(D)** The number of neurites per DRG. One-way ANOVA showed a significant variation among the treatment cohorts ($F(3, 32) = 4.433$, $p = 1.03 \times 10^{-2}$, with the ganglia cultured in the presence of a GDNF gradient showing a significantly higher number of neuronal processes than the control group.

These results indicate that the CNTF and GDNF induce directed neurite outgrowth when applied in combination, but not individually.

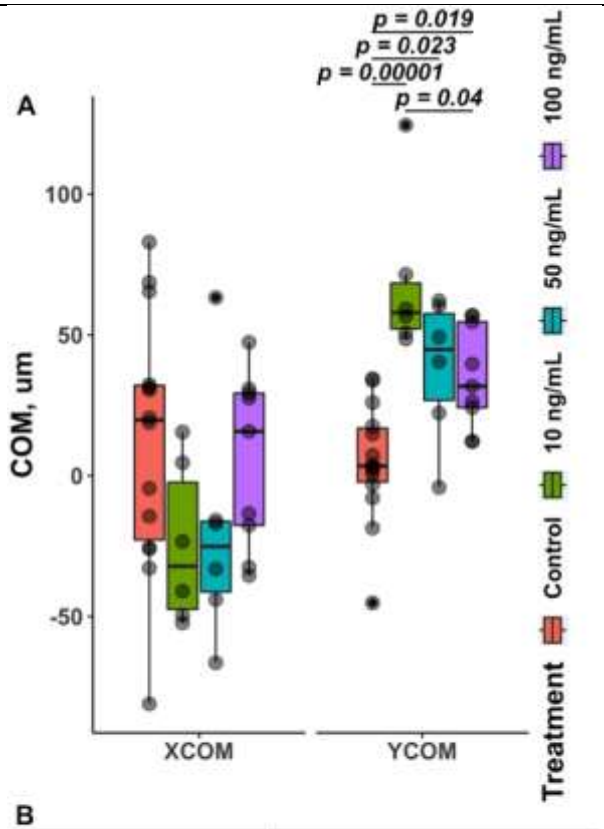
(5) The synergistic effect of CNTF and GDNF on directed growth in various concentrations

We previously tested the effect of neurotrophic factors combination (CNTF and GDNF) on directional neurite growth in various concentrations (0, 10, 50, and 100 ng/mL). The experimental groups include;

- Group 1. Control (no CNTF and GDNF)
- Group 2. CNTF + GDNF, 10 ng/mL each
- Group 3. CNTF + GDNF, 50 ng/mL each
- Group 4. CNTF + GDNF, 100 ng/mL each

Our data suggest that the combination of CNTF and GDNF sustains directed growth of DRG neurites towards the source of the chemotaxis gradients at all three concentrations. In all three treatments, the preferential extension of the neurites towards the source of the neurotrophic factors was reflected by a significant increase in the forward migration index (YFMI), as reported previously.

During this reported period, we corroborated this data by computing two additional metrics – 1) the center of mass displacement along the y-axis (YCOM) **(Fig. 7A)** (i.e., parallel to the gradient) and 2) average *p*-values of Raleigh test being below 0.05 **(Fig. 7B)**. Interestingly, there was no evidence of enhanced directed growth with increasing concentrations of the neurotrophic factors. The center of mass showed a significant displacement in the direction of the gradient source in all three concentrations **(Fig. 7A)**. Likewise, the p-value of the Rayleigh test was below 0.05 in all three treatment groups **(Fig. 7B)**.



B

Treatment	Rayleigh Test Average P-value
Control	0.225
CNTF+GDNF, 10ng/mL	0.033
CNTF+GDNF, 50ng/mL	0.014
CNTF+GDNF, 100ng/mL	0.001

Figure 7. Quantitative combined effect of different concentrations of the CNTF/GDNF mix (10 ng/mL, 50 ng/mL, and 100 ng/mL each) on directed growth of DRG neurites in the guidance assay after 48 hours in culture. **(A)** Center of mass (COM) displacement in µm along the x-axis (XCOM) and y-axis (YCOM). XCOM showed no statistically significant variation among the cohorts (One-way ANOVA $F(3, 31) = 1.691$, $p = 0.19$). YCOM varied significantly among the treatments (One-way ANOVA $F(3, 31) = 12.08$, $p = 2.09\times10^{-5}$). All three groups treated with the CNTF+GDNF gradients showed higher YCOM displacement values than the control group. The YCOM values in the group treated with the lowest concentration of CNTF and GNDF (10 ng/mL) were also significantly higher than the values in the group exposed to the highest concentration (100 ng/mL). **(B)** Average *p*-value of Rayleigh test. Note that only the three cohorts exposed to the gradient of the neurotrophic factors, but not the control group, showed values below 0.05.

We also evaluated the effect of neurotrophic factors combination (CNTF and GDNF) on the general growth metrics that are not directly related to the directed growth in various concentrations (0 (control), 10, 50, and 100 ng/mL each) **(Fig. 8)**. All four metrics (directness, the longest neurite length, average neurite length, and the number of neurites per ganglion) differentially reacted to specific concentrations of the neurotrophic factors.

The 100 ng/mL group showed a significantly increased directness ($p = 0.025$) compared to the control group **(Fig. 8A)**. The 50 ng/mL group showed a significantly increased value in the longest neurites ($p = 0.004$) compared to the control group **(Fig. 8B)**. The average neurite length significantly ($p = 0.019$) exceeded the control levels only in the 10 ng/mL group **(Fig. 8C)**. The number of neurites growing out from a given DRG was significantly (~2-fold, $p = 0.013$) higher in the 100 ng/mL group than the control **(Fig. 8D)**.

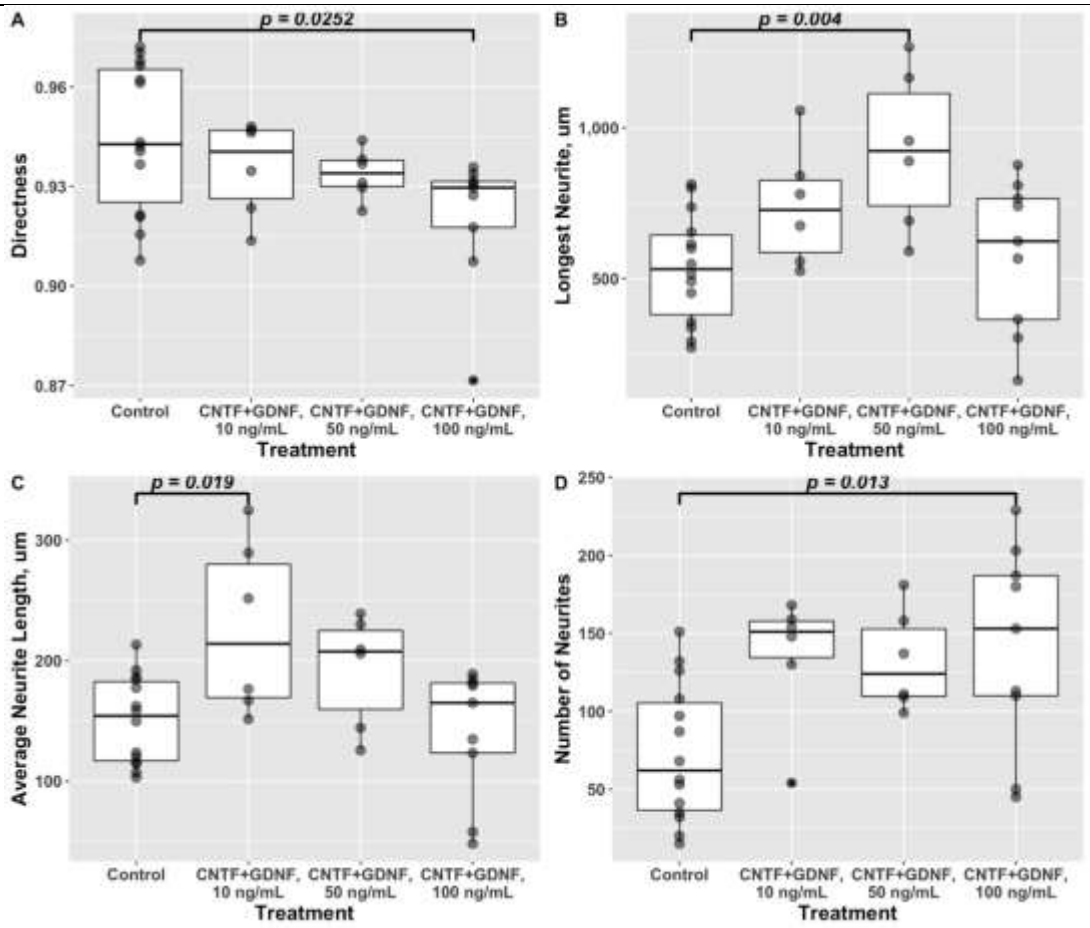


Figure 8. Quantitative effect of different concentrations of the CNTF/GDNF combination on growth properties of DRG neurites after 48 hours in culture. (A) Directness. Variation among the treatment groups was significant (one-way ANOVA $F(3,31) = 3.034$, $p = 0.044$) with the group treated with 100 ng/mL of CNTF and GDNF each showing a significantly lower value than the control group. (B) Length of the longest neurite in DRGs. There was a significant variation among the treatment groups (one-way ANOVA $F(3,31) = 5.232$, $p = 4.87\times10^{-3}$). The length of the longest neurite significantly increased in the cohort exposed to the intermediate concentration of CNTF/GNDF (50 ng/mL) compared to the control group. (C) Average neurite length. One-way ANOVA detected significant changes among the groups ($F(3, 31) = 4.698$, $p = 8.11 \times 10^{-3}$). The cohorts exposed to the lowest concentration of the combination of CNTF and GDNF (10 ng/mL each) showed a significant increase in the mean neurite length compared to the control group. (D) The number of neurites per DRG. One-way ANOVA showed a significant variation among the cohorts ($F(3,31) = 4.865$, $p = 6.9\times10^{-3}$), with the ganglia cultured in the presence of the highest concentration of the CNTF/GDNF mix (100 ng/ml) showing a significantly higher number of neuronal processes than the control group.

Our data demonstrate that a combination of two neurotrophic factors – CNTF and GDNF – induces directed growth of neurites with high efficiency and can facilitate innervation of implanted bioprinted muscle constructs in our subsequent experiments. We demonstrated that these two neurotrophic factors, when applied in a 1:1 combination, result in the directed growth of neuronal processes towards the source of the gradient. This chemotactic effect persists without significant changes over a wide (10-fold) concentration range. Moreover, we demonstrated that general growth parameters that do not evaluate growth in a specific direction (such as neurite length, number, and trajectory) were differentially affected by the concentration of the CNTF/GDNF combination. Furthermore, GDNF, when applied individually, did

not exhibit any chemotactic effect but caused significant neurite elongation and an increase in the number of neurites per ganglion.

(6) Neuronal outgrowth by the combination of CNTF and GDNF in the bioprinted muscle construct

We next tested if the combination of the two neurotrophic factors, CNTF and GDNF, that induced directional neurite growth in the 2D culture, would also facilitate neurite growth in the 3D bioprinted muscle constructs. In this pilot experiment, the CNTF + GDNF combination (10 ng/mL each) was incorporated into the bioink. The control constructs did not contain neurotrophic factors. Chick embryo DRGs were then placed on top of the constructs and cultured for 3 days. The co-cultured DRGs and constructs were then fixed and immunostained with an anti-neurofilament (NF) antibody and imaged using a confocal microscope.

The co-cultures containing neurotrophic factors in the bioink showed a much more robust neurite outgrowth into the underlying extracellular matrix of the bioprinted muscle construct than the control group (Fig. 9).

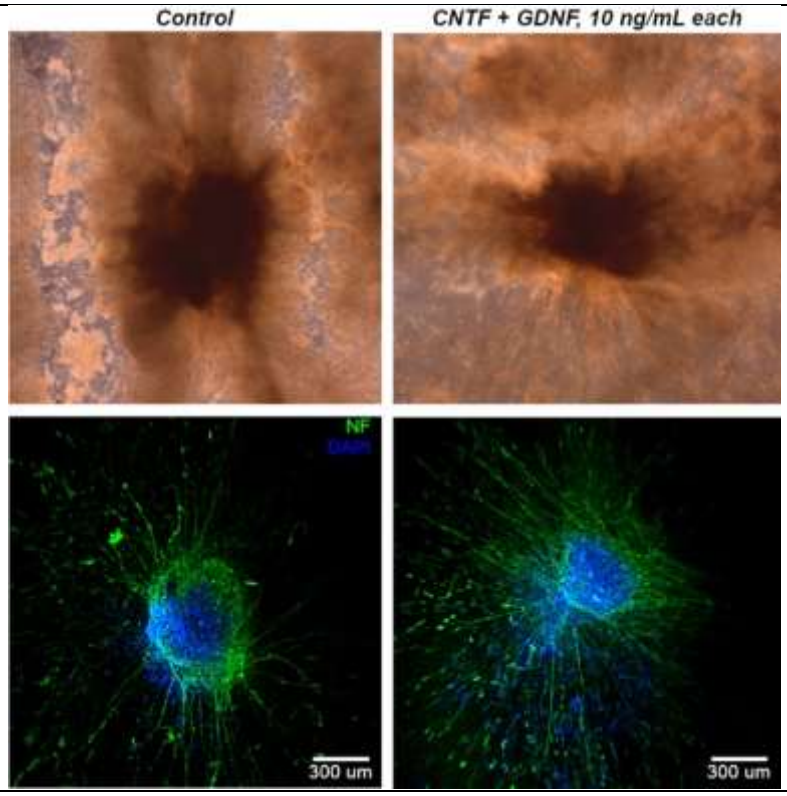


Figure 9. Effect of the combination of CNTF and GDNF on the innervation of the 3D printed construct by outgrowing neurites from co-cultured embryonic chick DRGs. CNTF and GDNF (10 ng/mL) each were incorporated into the bioink, and the DRGs were then placed on top of the construct. The constructs and the DRGs were co-cultured for 3 days, and immunostained with antibodies raised against neurofilament proteins. Cell nuclei were counterstained with DAPI. The control co-cultures contained no neurotrophic factors. Co-cultures containing CNTF and GDNF (right column) showed much more extensive neurite outgrowth than the control co-cultures (left column).

(7) Effect of the neurotrophic factors (CNTF and GDNF) on hMPCs viability and proliferation

We established that combining two neurotrophic factors - CNTF and GDNF – facilitates the directed growth of neurites towards the morphogen source. We intended to use these molecules in the bioprinted muscle constructs to ensure its timely and efficient innervation by the host's nervous system. However, the effect of those factors on hMPCs remains unknown. To this end, we tested how CNTF and GDNF would affect the viability and proliferation of hMPCs *in vitro*.

CNTF and GDNF were added to the cultured hMPCs either separately or in combination at 10 ng/mL each (The concentration of neurotrophic factor was optimized in terms of neurite outgrowth *in vitro* in our studies above). The viability and proliferation of the hMPCs were quantitatively assessed using the Alamar Blue Assay (Invitrogen) at day 1, 3, and 5 of the treatment. The data suggests that the neurotrophic factors had no deleterious effect on the cell growth and viability of the skeletal muscle progenitors **(Fig. 10)**.

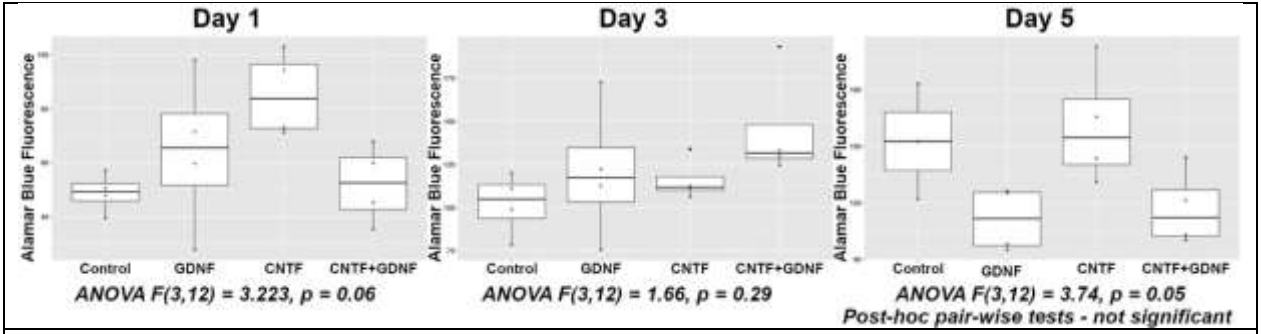


Figure 10. Effect of the neurotrophic factors CNTF and GDNF on the viability of hMPCs. The factors were applied at a concentration of 10 ng/mL, either individually or in combination. The cell health was assessed with the Alamar Blue cell viability assay. The data shows that the neurotrophic factors had no negative effect on the cell viability of the differentiating muscle progenitors.

(8) Fabrication of PLGA microspheres as a vehicle for controlled neurotrophic factor release in the bioprinted muscle construct

One of the crucial factors for the function of the implanted bioengineered muscle construct is its proper innervation. Therefore, it is critical to facilitate the growth of the host peripheral nerves and direct them towards the myotubes within the implanted muscle construct *in vivo*. This can be achieved if the implanted constructs can maintain a long-term gradient of neurotrophic factors that facilitate directed axonal growth. To this end, we developed a methodology to fabricate PLGA-based microspheres for controlled and sustained release of neurotrophic factors that will be incorporated into the bioprinted muscle construct.

PLGA is the most widely applied polymer in controlled release systems in tissue engineering and drug delivery contexts. The polymer composition and microsphere manufacturing protocol can be manipulated in a highly controlled way to achieve the desired properties of the delivery system. In the context of our project, the following properties of the microspheres are of particular importance:

i. size (should be compatible with the 3D bioprinting protocol)
ii. size distribution (should be narrow enough to ensure the release consistency of the active substances)

iii. encapsulation efficiency (should be sufficient enough to ensure an adequate load of neurotrophic factors)
iv. release kinetics (we aim for a long-term sustained release as opposed to a short burst release to ensure the presence of the neurotrophic factors in the construct for the entire amount of time required for the proper innervation of the implanted muscle)

We established a protocol that consistently yields microspheres meeting all the above requirements. This protocol was based on the water-in-oil-in-water (w1/o/w2) double emulsion technique. Chloroform was used as a solvent in the organic phase, which contained 10% PLGA (co-polymer ratio 50:50).

- The hydrophilic active ingredients (e.g., CNTF and GDNF) were dissolved in PBS (w1 phase).
- This solution was then emulsified in an organic phase (o) 10% PLGA (co-polymer ratio 50:50) in an organic solvent at a ratio of 1:100 by sonication at 50 W for 30 seconds.
- The primary emulsion was mixed with a second aqueous solution (w2) containing an emulsifier, such as 3% polyvinyl alcohol (PVA), at a ratio of 1:100.
- The organic solvent was removed by a continuous stirring (3 hours – overnight).
- The microspheres were collected from the remaining aqueous phase by filtration through a 100 µm cell strainer and centrifugation.

This protocol reproducibly produced homogeneously spherical microparticles, ranging in size between 10 - 40 µm (21.9 ± 1.4 µm, mean ± sd) with a high yield of 66.1 ± 2.8% (mean ± sd) **(Fig. 11A)**.

To demonstrate the PLGA microspheres' ability to encapsulate proteins, we determined the encapsulation efficiency with a direct method using bovine serum albumin (BSA) as a model protein. The microspheres were dissolved in 0.1N NaOH containing 5% SDS for 3 days, and the protein content was measured with the BCA Protein Assay kit. The encapsulation efficiency measured with this technique was 75.4 ± 12.6%. The encapsulated cargo molecules within the microspheres are presumably contained in numerous small spherical microcavities (average diameter 1.1 µm) that were evenly spaced throughout the internal volume of the microsphere **(Fig. 11B, C)**.

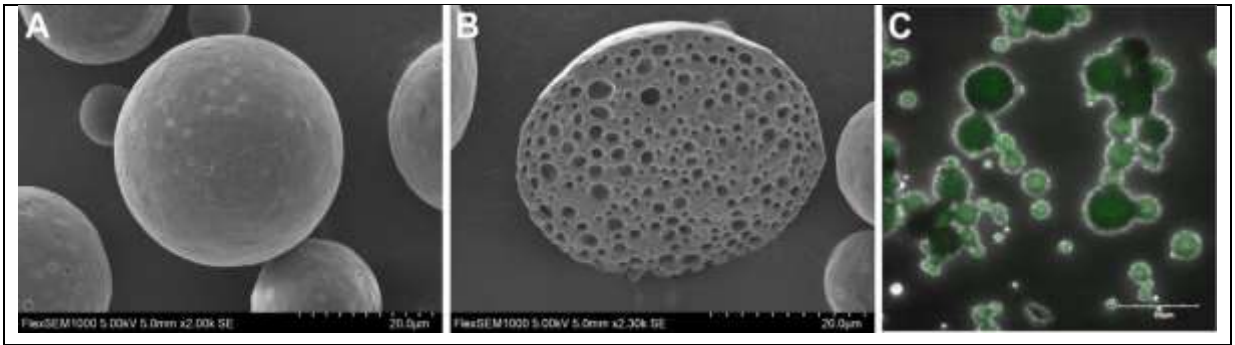


Figure 11. Morphology of PLGA microspheres. **(A - B)** Scanning electron microscopy of the external **(A)** and internal **(B)** morphology. Note spherical internal microcavities shown in **(B)**. **(C)** Soluble fluorescent dye fluorescein isothiocyanate-dextran (green)-encapsulated microspheres.

(9) Incorporation of PLGA microspheres into bioprinted skeletal muscle constructs

As described above, we incorporated the PLGA microspheres into the bioprinted muscle construct to enable delivery and sustain release of the neurotrophic factors CNTF and GDNF. It is uncertain whether

the bioprinted constructs would impose additional limitations on the microspheres. First, their addition to the bioink has to be compatible with bioprinting (i.e., they should not cause nozzle obstruction). Second, the presence of the microspheres in the hydrogel bioink should not compromise the health of the embedded muscle progenitor cells.

To this end, we tested the feasibility of using the PLGA microspheres as a delivery vehicle in our bioprinting system. The PLGA microspheres were mixed into the fibrin bioink containing hMPCs (30 M/mL) at the concentration of 5 mg/mL. The bioprinted skeletal muscle constructs were fabricated using the ITOP system. The microspheres-incorporated bioink was successfully dispensed without nozzle clogging, yielding the hydrogel structures similar to those produced with the microsphere-free bioink. The light microscopy image showed that the microspheres were evenly distributed within the hMPC-encapsulated bioprinted muscle structures **(Fig. 12A, B).**

The effect of PLGA microspheres on the viability of hMPCs in the bioprinted muscle construct was evaluated using the live/dead assay results. The bioprinted constructs without microspheres showed a high cell viability of 86.5% (n = 3). The viability of microspheres-incorporated bioprinted muscle constructs also showed a high cell viability of 87.8% (n = 3). There was no significant difference between the two groups **(Fig. 12A-C)**. These results indicate that the microspheres-incorporated bioprinted muscle construct can be fabricated without affecting the viability of the printed hMPCs.

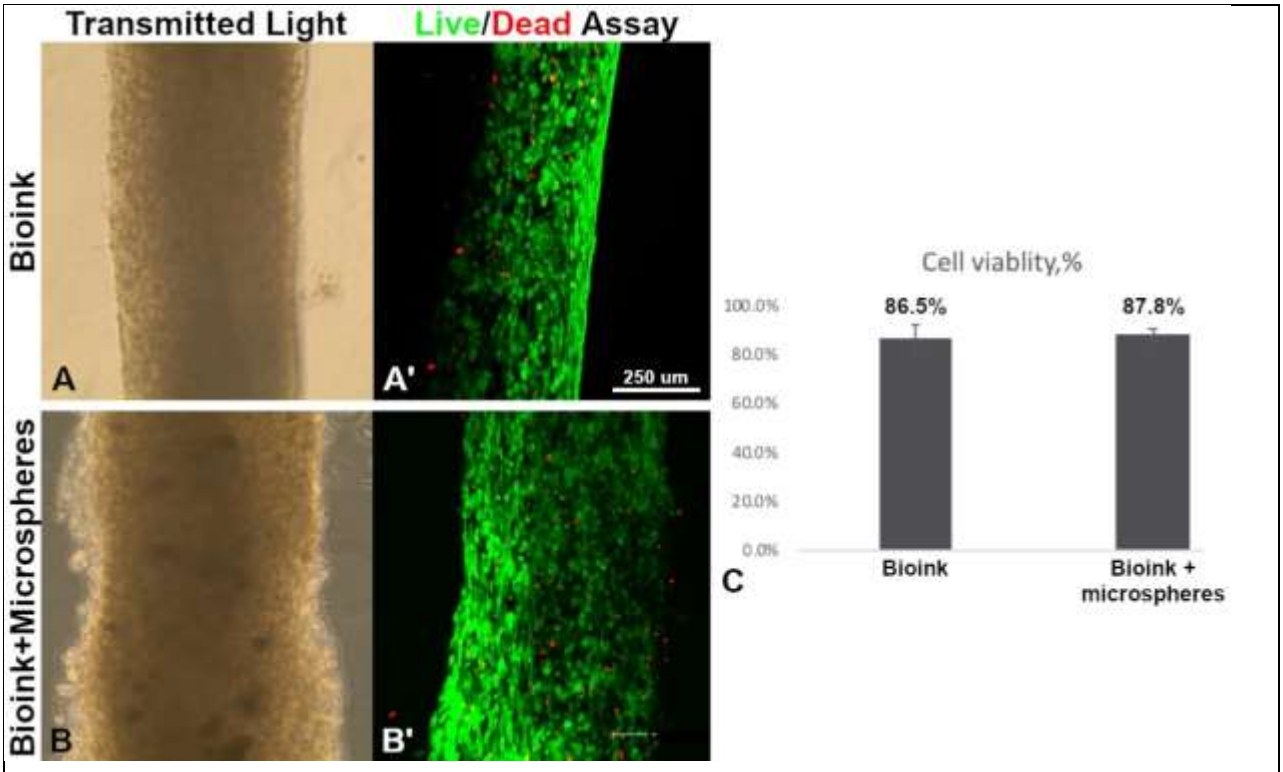


Figure 12. Effect of PLGA microspheres on the viability of hMPCs in bioprinted constructs. hMPC concentration is 30 million/mL. **(A and A')** Fibrin bioink without microspheres. **(B and B')** Bioink containing 5 mg/mL of PLGA microspheres. **(A and B)** Transmitted light micrographs. **(B and B')** Live/Dead assay (Molecular Probes). Z-projections of confocal stacks. Live cells are shown in green, and dead cell nuclei are shown in red. **(C)** Live cell percentage normalized to the total number of cells in the bioprinted constructs with and without microspheres. N = 3 per group.

(10) Sustained release of neurotrophic factors using the PLGA microspheres

Next, we tested if the microsphere-mediated delivery of the neurotrophic factors results in a more sustained presence of these factors than directly mixing them into the hydrogel scaffold of the construct (without microspheres). To this end, we manufactured two corresponding types of constructs:

- Group 1. Free CNTF/GDNF without microspheres (bioprinted muscle construct containing 0.5 µg/mL of CNTF and GDNF in the bioink)
- Group 2. Encapsulated CNTF/GDNF in microspheres (bioprinted muscle construct containing CNTF and GDNF-encapsulated microspheres in the bioink (0.5 µg/mL each))

The constructs were then incubated in the release buffer (PBS), which was collected for analysis on days 1, 3, 5, and 7. The released GDNF in the PBS was quantified with the ELISA assay. The neurotrophic factors encapsulated into the microspheres showed a significantly slower cumulative release than the neurotrophic factors freely dissolved in the bioink. Without microspheres, the amount of the released GDNF was continuously declinedfollowing the initial burst release at day 1. However, with microspheres, the amount of the released GDNF was stabilized after 5 days **(Fig. 13)**.

These data show that the PLGA microspheres can be used as a vehicle for controlled and sustained delivery of neurotrophic factors.

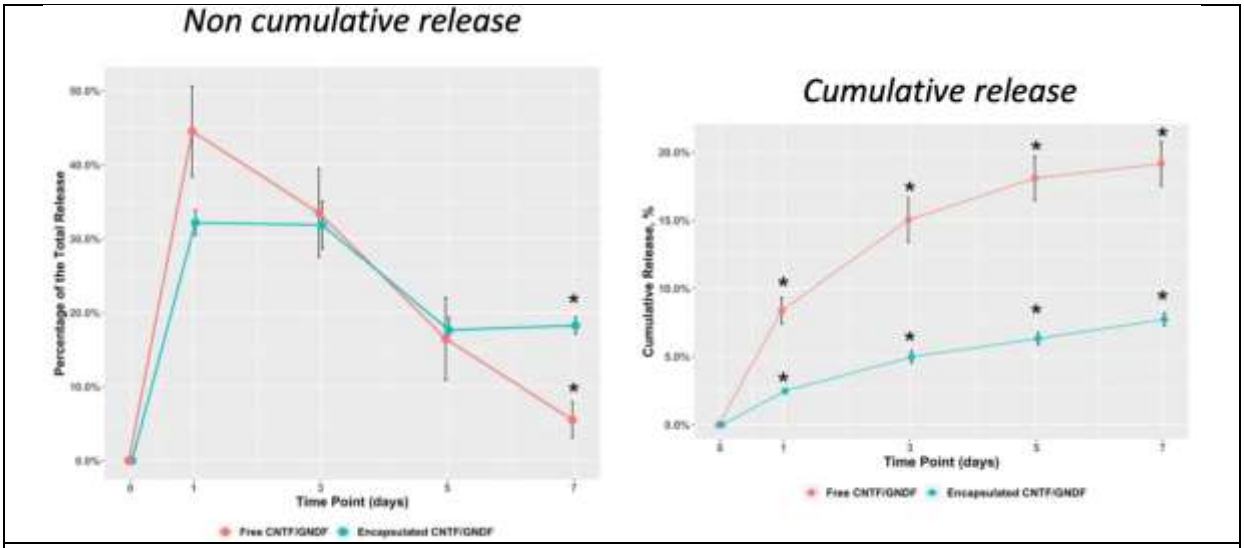


Figure 13. Release kinetics of GDNF from the bioprinted constructs containing freely dissolved CNTF/GNDF (0.5 µg per mL of bioink) and the construct containing the matching load of the same neurotrophic factors encapsulated into PLGA microspheres. The amount of the released GDNF was quantified by the ELISA Quantikine assay (R&D Systems). The data were processed and plotted in two complementary ways. Non-cumulative release (*left*) represents the percentage of the total release at a given time point and cumulative release percentage (*right*).

(11) NMJ formation by agrin and neurotrophic factors treatments

We have identified a combination of neurotrophic factors, CNTF and GDNF, as the efficient treatment to stimulate directional neurite outgrowth. We also established that the treatment of hMPCs with agrin facilitates the formation of AChR clusters. During this reporting period, we demonstrated that DRGs neurites co-cultured with hMPCs formed NMJs on the pre-formed receptor fields.

In this experiment, hMPCs were grown in 2D culture. The formation of AChR clusters was induced by treatment with agrin (150 ng/mL in DM) for 3 days, and then DRGs from 10 day-old chick embryos were added to the differentiated muscle fibers. The co-culture was maintained for 2 more days in the presence of agrin and neurotrophic factors (CNTF and GDNF, 10 ng/mL each). The samples were fixed and processed for immunocytochemistry with anti-NF and anti-AChR antibodies to visualize neurites and AChR clusters, respectively. The control groups included co-cultures incubated in pure DM, DM with agrin (no neurotrophic factors), and DM containing neurotrophic factors without agrin.

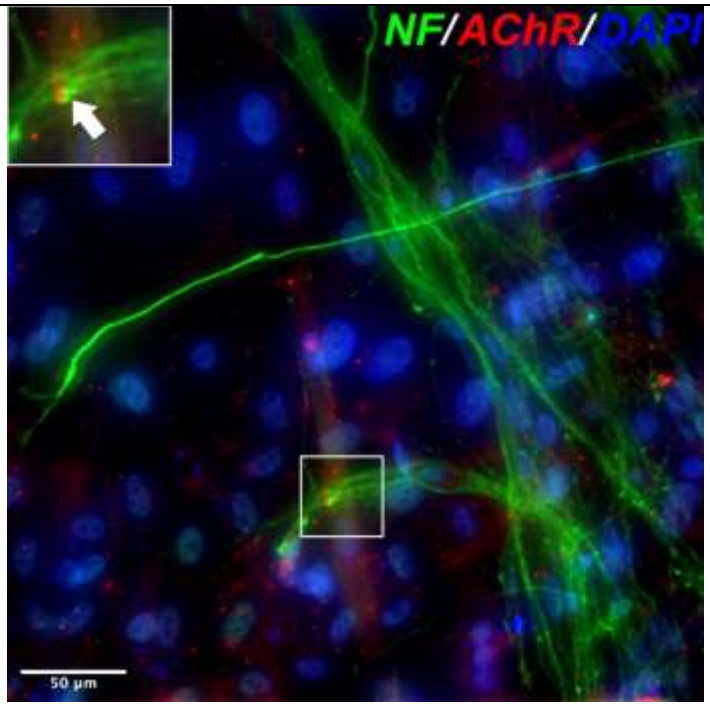


Figure 14. Neuromuscular junction (NMJ) between a neurite growing out from a chick embryonic DRG and a differentiated muscle fibers. The sample was treated with agrin to induce AChR cluster (red, arrow) formation on muscle fibers and a combination of two neurotrophic factors (CNTF and GDNF) to induce the neurite (green) outgrowth. The inset shows a high magnification of the boxed area in the main micrograph. NMJs between the neurites and AChR clusters on muscle fibers were observed.

Microscopic examination detected putative NMJs between the neurites and AChR clusters on muscle fibers (**Fig. 14**, white arrow: NF^+ and $AChR^+$ NMJs). These NMJs were more prevalent in the co-cultures treated with agrin and neurotrophic factors than in control groups. The result indicates that the agrin treatment can increase the AChR cluster pre-formation on the differentiated muscle fibers that enhances the NMJ formation with the nerve tissues.

This data demonstrates the feasibility of our two-pronged approach to facilitate innervation of differentiated skeletal muscle through both (a) enhancing neurite growth and (b) pre-formation of post-synaptic receptor fields on muscle fibers.

(12) A pilot *in vivo* study in an ectopic transposed nerve implantation model in rats

In our *in vitro* experiments, we have shown that the bioprinted muscle construct containing neurotrophic factor-loaded PLGA microspheres can be fabricated. Encapsulated CNTF and GDNF were released from the microspheres in a sustained manner. We successfully pre-formed the AChR clusters on human muscle fibers with agrin treatment. We also demonstrated the directional neural growth towards the intended target in the gradient of CNTF and GDNF *in vitro*. These results informed the design of the pilot *in vivo* study to confirm whether the controlled delivery of CNTF and GDNF using PLGA microspheres promotes the host neurite migration and ingrowth to the implanted muscle construct in the *in vivo* environment.

(a) Model creation

We tested the surgical procedures to implant a 3D bioprinted muscle construct in the rat transposed peripheral nerve model using rat cadavers. Briefly, a 20 mm-long incision was made on the popliteal skin of the right hind limb to expose the common peroneal nerve (CPN). The distal part of the nerve was wrapped around the subcutaneously implanted 3D printed muscle construct, and the construct was sutured to the underlying gluteus muscle to prevent displacement. The fascia layer was sutured to cover the implanted construct and the nerve. Lastly, the skin was closed using surgical staples **(Fig. 15)**.

The reliability of the animal model was confirmed. The surgical team had been trained on the procedures for consistency. On-line and hands-on training on implementing live animal surgeries were completed. The research team has practiced tissue sample evaluation techniques associated with EMG, specimen preparation, tissue sectioning, histology, immunohistochemistry, and imaging. In addition, the research team prepared cells, animals, materials, and supplies required for the *in vivo* studies.

(b) Implementation of in vivo implantation surgeries

After the completion of model creation and training required for the *in vivo* surgeries and evaluations, we initiated the pilot *in vivo* study. In this pilot study, we will investigate the effects of controlled and sustained release of neurotrophic factors using PLGA microspheres on accelerating host nerve integration *in vivo*. This study involves three experimental groups (n = 4 in each group):

- Group 1. Bioprinted muscle construct without neurotrophic factors (Control group) contains no extrinsic neurotrophic factors
- Group 2. Bioprinted muscle construct with free neurotrophic factors (contains 0.5 µg of CNTF and GDNF each per mL of bioink)
- Group 3. Bioprinted muscle construct with neurotrophic factors-encapsulated microspheres (the same amount of CNTF and GDNF-loaded microspheres were suspended in the bioink).

For the implantation, the bioprinted muscle constructs containing hMPCs (30 million/mL of bioink) and neurotrophic factors with/without microspheres were fabricated. The pre-formation of AChR clusters was induced by incubation of the bioprinted muscle constructs in DM containing agrin (150 ng/mL) before implantation according to our established protocol. The bioprinted muscle constructs were implanted into the transposed CPN model in athymic male rats (Charles River, body weight 250 - 300 g) **(Fig. 15).**

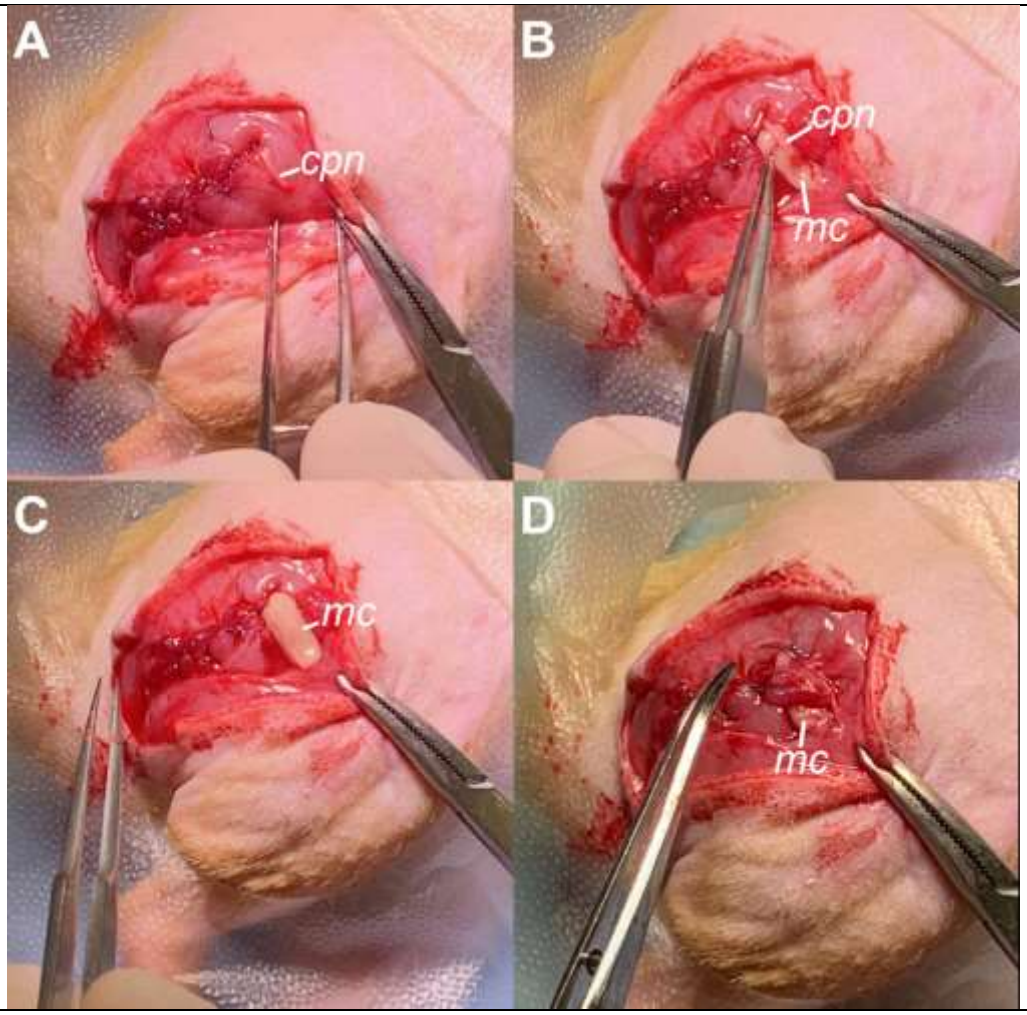


Figure 15. Transposed nerve model, implantation surgery. **(A)** The distal end of the common peroneal nerve *(cpn)* is dissected out and place on the surface of the gluteus muscle. **(B)** The superficial fascia of the gluteus muscle is opened and one half of the bioprinted muscle construct *(mc)* is placed on top of the muscle. The distal end of the CPN is placed on top of the construct. **(C)** The distal end of the CPN is overlaid by the second half of the construct so that the nerve is sandwiched between the two layers of the bioprinted muscle construct. **(D)** The implanted construct is covered with the facia, which is sutured in place.

The *in vivo* implantation surgeries are currently in progress. Four weeks post-implantation, the muscle function will be evaluated by EMG. The tissue samples will be harvested and snap-frozen in liquid nitrogen for histological and immunohistochemical analysis.

2. Future Plans

In the third year of the project, we will be focusing on the following tasks:

Task 1. Development and validation of bioprinted skeletal muscle constructs with NMJ formation capacity and neurotrophic factor release system

Task 1.1. Determine the effects of neurotrophic factors on skeletal muscle development and acetylcholine receptor (AChR) cluster pre-formation on the bioprinted muscle construct.

We will further optimize the parameters for the incorporation of PLGA microspheres into the bioprinted muscle construct. We will investigate the bioactivity of the neurotrophic factors released from the PLGA microspheres in terms of neurite outgrowth.

Task 1.2 Demonstrate NMJ formation and innervation on the bioprinted skeletal muscle constructs *in vitro.*

We will quantitatively analyze the innervation efficiency of the bioprinted muscle construct by using DRG neurites.

Task 1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model.

We will continue the *in vivo* implantation surgeries and evaluations (EMG study, histology).

Task 2. Determination of the clinical feasibility of restoring muscle function using bioprinted skeletal muscle constructs and nerve grafts in a preclinical traumatic muscle injury model.

Task 2.1 Create a nerve-muscle injury model in rats.

We will develop a relevant surgery procedure, evaluate its reproducibility, and train the team members using rat cadavers.

Task 2.2 Evaluate structural and functional recovery with NMJ formation.

We will prepare materials and supplies necessary to initiate the *in vivo* implantation surgeries.

3. Problems / Issues

a. Current Problems / Issues

This year, our institution has been severely affected by the on-going COVID-19 pandemic. Between late February and early June, we had to follow the implemented social distancing policies that resulted in the closure of our laboratories, which caused the cessation of our benchtop research activities. The laboratories were re-opened in early June, and our research experiments have resumed with a gradual

increase in activities. However, the effects of COVID-19 are escalating, and the prolonged COVID-19 pandemic has delayed and impacted the progress of the tasks in this project.

b. Anticipated Problems / Issues

After returning to the lab, we are required to follow social distancing guidelines, which limits the number of researchers working at the same time in the lab areas. We have put in place personnel and time management strategies that allowed us to resume our research activities at the level closer to the pre-pandemic rate. However, we anticipate that the on-going COVID-19 pandemic will further delay the progress and impact the entire tasks in this project. Therefore, we requested a No Cost Extension (NCE) of 6-month to complete tasks in this project, which is currently under review by Army contracting.

4. Financial Health

Before the cessation of experiments caused by the COVID-19 pandemic, the project was on track to meet the major milestones. After re-opening the lab in June, the research team is fully assembled. Currently, the PI and all team members continue to process the data and quantitative analyses, perform *in vitro* and *in vivo* experiments, and manuscript writing. The research team regularly tracks the progress of the project through weekly all-hands meetings

5. Personnel Effort

Personnel	Role	Percent Effort
Yoo, James	Principal Investigator	15%
Jackson, John	Co-Investigator	15%
Ju, Young Min	Co-Investigator	15%
Kim, Ickhee	Co-Investigator	20%
Kim, Ji Hyun	Co-Investigator	30%
Lee, Sang Jin	Co-Investigator	15%
Vaughan, Bill	Co-Investigator	5%
Cranfill, Jeff	Lab Technician	10%
Kim, Michael	Lab Technician	100%
Lim, Diana	Graduate Student	40%
Mashanov, Vladimir	Research Associate	100%
Perez, Jovanna	Lab Technician	50%
Young, Anna Marie	Lab Technician	10%

6. Protocol and Activity Status

a. Human Use Regulatory Protocols

TOTAL PROTOCOLS: 1 (one) human use protocol is required to complete this project.

PROTOCOLS:

Protocol: IRB00051342
Title: Discarded skeletal muscle tissue for use in laboratory cell processing and scaffold development
Target required for clinical significance: 30
Target approved for clinical significance: 30
Submitted to and Approved by:

- Submitted to HRPO on 01/16/2019
- Approved by HPRO on 08/14/2019

STATUS: Approved.

b. Use of Human Cadavers for RDT&E, Education or Training

TOTAL ACTIVITIES: No RDT&E, education or training activities involving human cadavers will be performed to complete the Statement of Work (SOW).

c. Animal Use Regulatory Protocols

TOTAL PROTOCOLS: 1 (one) animal use protocols is required to complete this project

PROTOCOLS:

Protocol: A18-153
Title: Accelerated Innervation of Engineered Tissue Construct
Target required for statistical significance: 1344
Target approved for statistical significance: 1344
Submitted to and Approved by:

- IACUC protocol #A18-153 was approved on 12/12/2018.
- Amendment #1 for IACUC study #A18-153 was approved on 12/18/2018.
- Amendment #2 for IACUC study #A18-153 was approved on 12/28/2018 (personnel change request, ACURO approval is not needed).
- ACURO appendix for IACUC protocol A18-153 was submitted to ACURO on 1/15/2019.
- Additional information for IACUC protocol A18-153 was submitted to ACURO on 4/9/2019 per ACURO's request.
- ACURO approved on 4/30/2019
- Amendment #3 for IACUC study #A18-153 was approved on 07/08/2019 (personnel change request, ACURO approval is not needed).
- Amendment #4 for IACUC study #A18-153 was approved on 09/04/2019 (personnel change request, ACURO approval is not needed).
- Amendment #5 for IACUC study #A18-153 was approved on 10/02/2019 (personnel change request, ACURO approval is not needed).
- Amendment #6 for IACUC study #A18-153 was approved on 02/06/2020 (ACURO approved on 2/13/2020)

- Amendment #7 for IACUC study #A18-153 was approved on 03/02/2020 (ACURO approved on 3/10/2020)
- Amendment #8 for IACUC study #A18-153 was approved on 03/18/2020 (personnel change request, ACURO approval is not needed).
- Amendment #9 for IACUC study #A18-153 was approved on 03/24/2020 (Withdrawn from ACURO after the communication with ACURO)
- Amendment #10 for IACUC study #A18-153 was approved on 04/07/2020 (personnel change request, ACURO approval is not needed).
- Amendment #11 for IACUC study #A18-153 was approved on 06/25/2020 (ACURO approved on 07/07/2020)

STATUS: Approved

Annual Business Status Report for

Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System Research

Research Project No. 2017-614-002

EGS# MT180011

Reporting Period: 10/01/2019 – 09/30/2020

MTEC Research Project Awardee

James Yoo

John Jackson, Young Min Ju, Ickhee Kim, Ji Hyun Kim, Sang Jin Lee

Research Project Technical POC

James Yoo

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Winston Salem, NC 27107

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jyoo@wakehealth.edu

Submitted: 10/25/2020

1. CURRENT STAFF

Personnel	*% of Effort on project*
James Yoo: Principal Investigator	15%
John Jackson: Co-Investigator	15%
Young Min Ju: Co-Investigator	15%
Ickhee Kim: Co-Investigator	20%
Ji Hyun Kim: Co-Investigator	30%
Sang Jin Lee: Co-Investigator	15%
Bill Vaughan: Co-Investigator	5%
Jeff Cranfill: Technician	10%
Michael Kim: Technician	100%
Diana Lim: Graduate Student	40%
Vladimir Mashanov: Research Associate	100%
Jovanna Perez: Technician	50%
Anna Marie Young: Technician	10%

2. CURRENT EXPENDITURES

A. Cost Reimbursable Contracts

Contract Expenditures	Current Annual Expenditures	*Cumulative To Date Expenditures*
Labor (Personnel and Fringe)	$ 60,441.58	$ 433,934.05
Supplies/Materials	$ 10,210.37	$ 77,915.00
Travel	$	$ 9,779.04
Equipment	$	$
Subcontractors and Consultants	$	$
Other Direct Costs	$ 33,360.96	$ 148,929.63
Indirect Costs	$ 57,207.11	$ 368,806.76
Total	$161,220.02	$1,039,364.48

B. Fixed Priced Contracts: n/a

C. Cost Share Contributions: n/a

3. STATUS OF MILESTONES

MTEC Milestone Number	Milestone Description	Due Date	% Completed this Reporting Period	Cumulative % Complete
1	Project Kick Off	10/15/2018		100%
2	Quarterly Report 1 (October - December, Technical and Business Reports)	01/25/2019		100%
3	Quarterly Report 2 (January - March, Technical and Business Reports)	04/25/2019		100%
4	Quarterly Report 3 (April- June) Technical and Business Reports)	07/25/2019		100%
5	Annual Report 1	10/25/2019		100%
6	Quarterly Report 4 (October-December, Technical and Business Reports)	01/25/2020		100%
7	Quarterly Report 5 (January - March, Technical and Business Reports)	04/25/2020		100%
8	Quarterly Report 6 (April-June, Technical and Business Reports)	07/25/2020	100%	100%
9	Annual Report 2	10/25/2020		
10	Task 1.1/D1.1 Determine the effects of neurotrophic factors on skeletal muscle development and acetylcholine receptor (AChR) pre-formation on the bioprinted muscle construct.	12/25/2020	7%	80%
11	Task 1.2/ D1.2 Demonstrate NMJ formation and innervation in bioprinted skeletal muscle constructs, in vitro.	12/25/2020	7%	80%
12	Quarterly Report 7 (October-December, Technical and Business Reports)	01/25/2021		
13	Quarterly Report 8 (January - March, Technical and Business Reports)	04/25/2021		
14	Task 1.3/D1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model.	06/25/2021	18%	47%
15	Quarterly Report 9 (April - June, Technical and Business Reports)	07/25/2021		
16	Task 2.1/D2.1 Create a nerve-muscle injury model in rat.	09/25/2021		
17	Annual Report 3	10/25/2021		

18	**Quarterly Report 10 (October-December, Technical and Business Reports)**	**01/25/2022**		
19	**Quarterly Report 11 (January - March, Technical and Business Reports)**	**04/25/2022**		
20	**Task 2.2/D2.2 Evaluate structural and functional recovery with NMJ formation.**	**05/30/2022**		
21	**Task 3.1/D3.1 Develop and validate BRs and SOPs to support a Pre-IND application.**	**05/30/2022**		
22	**Final Technological and Business Report**	**05/30/2022**		

4. DEVIATION FROM PROJECT PLAN

None