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Investigating Alternative Measures of Functional Recovery in Rat Sciatic Nerve Injury

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INTRODUCTION

There is a pressing need for advancements in peripheral nerve repair techniques and recovery evaluation methods. High rates of peripheral nerve injury incidence combined with poor functional outcomes are the main drivers of novel research in this arena. In developed countries, between 13 and 23 per 100,000 new peripheral nerve injuries occur yearly [1]. Primarily young, active people suffer peripheral nerve damage, causing them to incur lifelong disability and loss of economic productivity for the remainder of their lives.

Although autografts are the current gold standard for peripheral nerve recovery, they frequently result in negative outcomes. Healthy nerves must be sacrificed from a limited availability of viable tissue [2]. At the harvest site, scarring, sensory loss, morbidity, and neuroma occur [3]. At the donor site, additional incisions are required to insert the autograft, further damaging debilitated tissue [1]. Incongruencies between nerves frequently result numbness and functional recovery far below expectations [2, 4].

Other methods of repair such as allografts, mesh inserts, and muscle grafting do not show as promising results as recent advancements in tissue engineered conduits [1]. Improved and validated testing methods will determine the repair methods that result in the best functional return.

The rat sciatic nerve injury model is well examined in literature as a model for peripheral nerve repair. Histomorphological and electromyographic evaluations of nerve repair are common, and include axon counts, average axon diameter, motor unit counts, muscle weight, and muscle reinnervation via electromyograms [5, 6]. While useful in studying the processes of repair, it is well documented that these methods often do not correlate with functional outcomes [5, 6, 8].

Evaluation of the Sciatic Functional Index (SFI) is the current standard for functional through the analysis of the animal's gait. Walking track analyses like SFI are favorable because they evaluate sensory and motor neuron functional return. SFI is calculated from footprint length and toe spread in the middle of the gait cycle [8]. While SFI is an accurate measure of functionality for rats with normal toe spread, its validity has been challenged in the presence of certain conditions. SFI fails in the presence of autotomy (self-mutilation) and toe contractures (toe curl) in which the rat may walk on only the heel of the foot or on top of its curled toes [9, 10]. In one study, 80% of an experimental group exhibited toe contracture, decreasing the impact of the experiment [8]. Further, SFI fails to account for changes in velocity mid-trial and between rodents. Print length, the main component of SFI, has been shown to vary significantly with speed [11]. While there are other novel functional assessment methods in literature, validation is required to better evaluate the results and differences. No one test may universally indicate recovery, but further investigation can prove which are more accurate for certain targets and populations.

METHODS

Experimental Design

The purpose of this study is to compare new walking track evaluation methods applied to a previous animal study. In the previous study, nanofiber conduits for peripheral nerve repair were evaluated in a rat sciatic model. Conduits were created from both aligned Arginylglycylaspartic acid-poly(ε-caprolactone) (RGD-PCL) peptide functionalized nanofibers and non-functionalized PCL control nanofibers, seen in *Figure 1.* Details regarding the creation of the nanofiber conduits and execution of the animal study are currently in submission [12].

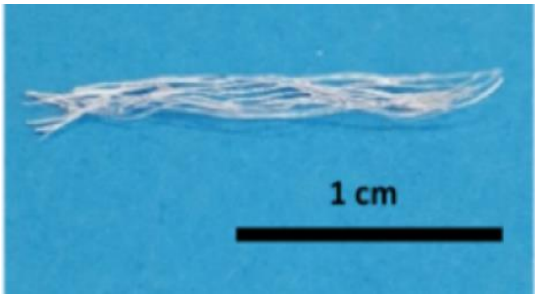

Figure 1. Image of the electrospun nanofibers prior to being functionalized provided by Cavanaugh et al., 2019 [12]

The scope of this project entailed the functional assessment methods without handling any animals. In this IACUC-approved study to model peripheral nerve repair, gaps were created in the sciatic nerves of male Lewis rats. One hind leg served as a sham and the other as an experimental. The rats were randomly assigned to four experimental groups for nerve repair, with five animals per group: repair via an isograft, an empty conduit (negative control), the RGD-PCL fiber and a PCL control fiber. Functional recovery was assessed through SFI biweekly. The current study served to retrospectively extract data to compare four walking track analysis methods:

1. Sciatic Functional Index
2. Imbalance Coupling
3. Stance Factor
4. Toe Out Angle

While these tests were not novel individually, their side-by-side comparison has not been performed. I investigated these evaluation techniques to may reveal which are correlated, which are least subject to variation, and if any had the potential for a new industry standard for evaluating nerve repair.

Sciatic Functional Index (SFI)

Images were taken while the target foot was in midstance and the other foot was mid-swing in the gait cycle. Three measures were used to calculate SFI: print length, the distance from the heel to the third toe; toe spread, the distance from the first to the fifth toe; and intermediate toe spread, distance from the second to the fourth toe. A healthy SFI should hover near 0, while scores closer to -100 indicate total impairment. Examples of both healthy and impaired SFI can be seen in *Figure 2.* SFI is expressed as a ratio of the experimental foot measurements to the sham foot measurements. SFI was calculated by Equation 1, where PLF, TSF, and ITF are the percent change from sham to experimental print length, toe spread, intermediate toe spread [13].

$$SFI = (-38.3 \times PLF) + (109.5 \times TSF) + (13.3 \times ITF) - 8.8 \quad (1)$$

Imbalance Coupling (IC)

Bozkurt, et al., 2011 [7] defined coupling as the “percentage of the step cycle of a certain paw (the anchor paw) at which the step cycle of another paw (the target paw) commences.” The gait cycle of a single foot commences with heel strike and is completed at the following heel strike of the same foot. This measure required a minimum of four

consecutive steps at a constant speed. A healthy score ranges from 0.48-0.52, indicating that a new step cycle begins halfway through the other foot's cycle. Serradj and Jamon, 2009 [14], showed that hind and front leg coupling patterns vary significantly with walking speed. This functional measure should be independent of abnormal gait, autotomy, or toe contracture. IC was an individual measurement per foot. To compare IC to the other evaluation methods it was expressed as a ratio of the sham to experimental foot. Thus, the optimal value was 1, which indicated the experimental foot is equal to sham levels.

Figure 2. Toe spread in a sham and experimental foot two weeks after sciatic nerve damage. The toe spread in the sham foot is representative of near 0 SFI, while the toe spread in the experimental foot is representative of near -100 SFI.

Stance Factor (SF)

Stance Factor is the ratio of the duration of ground contact between the uninjured and injured feet. required a minimum of four consecutive steps at a constant speed. This measure required a minimum of four consecutive steps at a constant speed. Stance factor is low for injured rats and steadily increases toward a 1 with recovery, indicating the rats are spending equal amounts of time on each foot. This measure has been shown to correlate with SFI and is a good alternative because abnormal gait, autotomy, and toe contracture do not interfere with the measurement [11].

Toe Out Angle (TOA)

Toe out angle is the angle between the direction of progression and a reference line in through the heel of the foot and the third digit. Varejao et al., 2004 [10] noted this measure correlated well with SFI in measuring functional recovery. Healthy rats exhibit low angles of just a few degrees. The angle is expected to be higher post-injury and slowly decrease to pre-injury levels with healing. Increased toe out angle has been shown to correlate significantly with increased walking speed [14]. This measure is a good alternative to SFI because it can still be calculated in instances of autotomy and toe contracture. TOA was an individual measurement per foot. To compare TOA to the other evaluation methods it was expressed as a ratio of the sham to experimental foot. Thus, the optimal value was 1, which indicated the experimental foot is equal to sham levels.

Statistical Analysis

One way repeated measures ANOVA tests were conducted in Minitab® to view statistical significance for each evaluation method. Significance was considered $p<0.05$ and can be found in *Table 1*. The first ANOVA test excluded week 12 to include the isograft group. The second ANOVA test excluded the isograft group to include week 12. The factors were the week, treatment method, and subjects. Tukey pairwise comparison identified which groups caused significant differences been factors. A Pearson correlation was used to determine the correlation between evaluation techniques. A Pearson coefficient between the absolute values of 1 and 0.7 indicated a strong relationship. Significance was considered $p<0.05$.

RESULTS

Walking track videos were obtained at 2, 6, and 12 weeks post-surgery for the RGD, PCL, and Empty Conduit groups. In addition, video captures from the isograft group were analyzed at 0, 2, and 6 weeks post-surgery. Gait abnormalities and poor video quality reduced the number of animals available for all four evaluation methods. In the original study, there were n = 20 in the RGD and PCL groups, n = 21 in the empty conduit group, and n= 12 in the isograft group. Toe contracture in prevented SFI evaluations in every group. Toe contracture was present in RGD n= 2, PCL n = 2, isograft n = 10, empty conduit n = 1. Missing or poor video quality excluded more animals from this study in isograft n = 6 and empty conduit n = 7 groups. These reductions in available videos resulted in each group having n = 5 animals, which decreases the impact of the experiment.

The ranges and means of the four evaluation methods for each group over time is presented in *Figure 2*. In SFI, each treatment group in weeks 0, 2, and 6 had a small range relative to the scale of the measurement. In week 12, the ranges of the PCL and RGD groups increased. For example, the standard deviation of the RGD group in week 0 was 4.9, while the standard deviation for week 12 was 21.1. SFI can be seen to increase over time for every group, indicating recovery.

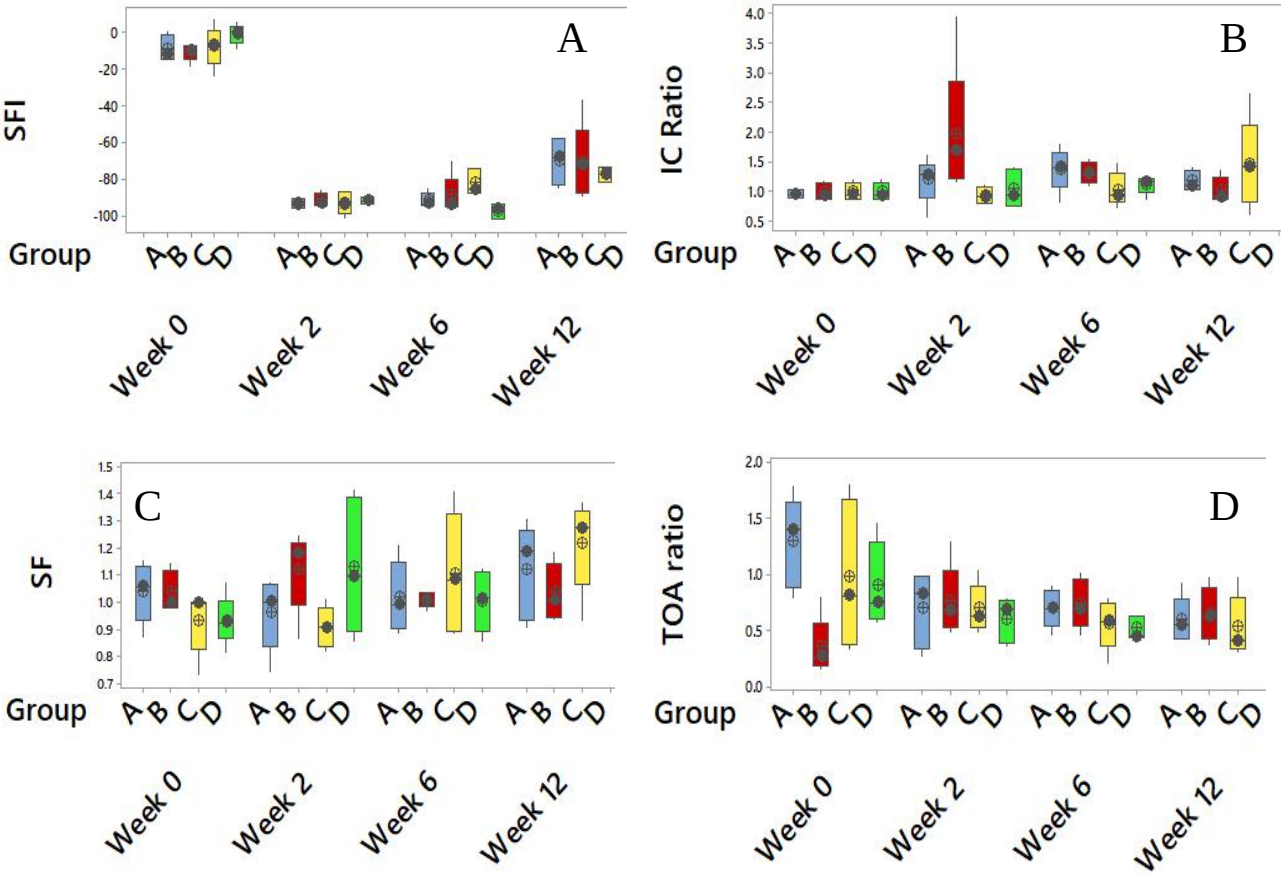

Figure 2. Ranges and means of treatment groups over 12 week trial. The isograft group was evaluated over 6 weeks, while the RGD, PCL, and empty conduit groups were evaluated over 12 weeks. A - Sciatic Functional Index. B - Imbalance coupling. C - Stance factor. D - Toe out angle

Table 1. Analysis of variance for each evaluation method. Factors tested include treatment method (RGD, PCL, empty conduit, and isograft), week (0-12), and subject. Significance was considered $p<0.05$. Weeks 0 - 12 did not include the isograft group, while weeks 0 - 6 included the isograft group.

Weeks 0 - 6			Weeks 0 - 12		
Source	Factor	p-value	Source	Factor	p-value
SFI	Treatment	0.518	SFI	Treatment	0.957
	Week	0.000		Week	0.000
	Subject	0.634		Subject	0.692
IC	Treatment	0.030	IC	Treatment	0.408
	Week	0.102		Week	0.205
	Subject	0.735		Subject	0.414
SF	Treatment	0.419	SF	Treatment	0.912
	Week	0.559		Week	0.093
	Subject	0.852		Subject	0.694
TOA	Treatment	0.111	TOA	Treatment	0.206
	Week	0.098		Week	0.655
	Subject	0.824		Subject	0.655

In IC, most groups had relatively low variability, like the PCL group in week 0, with a mean and standard deviation of 0.95 ± 0.07. The RGD group in week 2 and the empty conduit group in week 12 had higher variability. The mean and standard deviation for the RGD group in week 2 was 1.95 ± 1.14, while the mean and standard deviation of the empty conduit group in week 12 was 1.5 ± 0.78. In SF, most groups over every time point had a large range relative to the scale of the measurement. The lowest standard deviation was in the RGD group in week 6, at 1.01 ± 0.03. Contrastingly, the standard deviation was 5 times higher in the same group in week 2 at 1.12 ± 0.15. In TOA, pre-surgery evaluations had generally higher means and standard deviations than later evaluations. In the empty conduit group in week 0, the mean and standard deviation was 0.98 ± 0.66. In the isograft group in week 6, the mean and standard deviation were 0.51 ± 0.1.

In a regression model, SFI over time had an R-squared value of 94.5%. IC, SF, and TOA had low R-squared values of 31.8%, 27.6%, and 28.9%, respectively.

In a Pearson correlation, shown in *Table 2*, imbalance coupling correlated significantly with SFI in both the 6 week and

12 week trials. Stance factor and imbalance coupling were correlated significantly in both the 6 and 12 week trials. Interestingly, although IC correlated with both SFI and SF, SF did not correlate with SFI. Stance factor and imbalance coupling may correlate because both measures were calculated from the exact same measurements of the videos.

No significant difference was found between treatments or subjects in SFI, SF, or TOA. For SFI, 6 and 12 week trials both saw significant increases over time (p=0.00 for both). A Tukey pairwise comparison indicated that the Week 0 differed significantly from weeks 2, 6, and 12. Weeks 2 and 6 were not significantly different, but week 12 was significantly different from the other weeks. This significant increase in SFI indicates recovery. For IC in the 6 week trial, a significant difference was found between treatment groups for all weeks and subjects (p=0.03). No significant difference was found between weeks or subjects. A Tukey pairwise comparison indicated a significant increase in the RGD group compared to the other groups. Pearson correlations examined for the 6 and 12 week trials were detailed in *Table 2*.

Table 2. Pearson correlation coefficients and p-values define the degree of correlation between evaluation techniques. Weeks 0 - 12 did not include the isograft group, while weeks 0 - 6 included the isograft group. Significance was considered $p<0.05$.

Weeks 0-6					Weeks 0-12				
Treatment	Measure	SFI	IC	SF	Treatment	Measure	SFI	IC	SF
IC	Pearson coefficient	-0.28			IC	Pearson coefficient	-0.27		
	p-value	0.03				p-value	0.04		
SF	Pearson coefficient	-0.14	0.26		SF	Pearson coefficient	-0.08	0.4	
	p-value	0.29	0.05			p-value	0.54	0.00	
TOA	Pearson coefficient	0.29	0.01	-0.03	TOA	Pearson coefficient	0.21	-0.09	-0.02
	p-value	0.02	0.97	0.8		p-value	0.11	0.51	0.86

When evaluating up to 6 weeks of post-surgery, imbalance coupling was significantly different between treatments. A Tukey pairwise comparison indicated a significant increase between the RGD group and the other groups in week 2. The RGD animals had a wide variation (1.95 +/- 1.14), and this variation came almost entirely from one animal, despite not statistically qualifying as an outlier. Removing this animal brought the mean and standard deviation of the RGD group to 1.45 ± 0.29, which would be the highest average of any group. The next highest mean and standard

deviation was 1.2 ± 0.18 in the PCL group. It is reasonable to dismiss the significant increase between treatments in IC to be due to the one animal.

DISCUSSION

In this study, four techniques were employed to quantify the functional return after peripheral nerve damage. The rat sciatic model was used to compare the nerve repair potential of two nanofiber conduits to an empty conduit and the current gold standard, an isograft. Video recordings of the animals on a walking track allowed for the evaluation of the sciatic functional index, imbalance coupling, stance factor, and toe out angle of the animals. Walking track analyses were conducted pre-surgery and 2, 6, and 12 weeks post-surgery. Sciatic functional index, the current standard for quantifying functional recovery, fails in certain conditions common to the rat sciatic model [9, 10]. While SFI fails in the presence of autotomy and toe contractures, imbalance coupling, stance factor, and toe out angle do not [7, 11, 10]. Gait velocity was not included as a covariate in the scope of this project. This study served to assess the value of the other walking track evaluation methods by determining which are correlated.

Walking speed may be a confounding variable that affected SFI, imbalance coupling, and TOA. SFI has already been shown to vary significantly with speed in other studies [11]. Print length, the main component of SFI, is the main cause of this variation. Higher walking speeds result in shorter print lengths. Decreased print length correlates with lower SFI, indicating a return to healthy gait [11] This correlation would indicate that an animal walking slower in one trial has a more impaired gait than the same animal in another trial walking at a higher velocity. The problem arises, however, when considering factors not related to recovery that cause slow walking speed, like fatigue or the exploration of an unfamiliar area. Because of these flaws in SFI, this measure can misrepresent level of recovery when speed is not controlled. Contrasting with SFI, TOA has been shown to increase with walking speed [14]. While higher walking speed is correlated with recovery in SFI, it correlates with impairment in TOA. Coupling patterns between front and hind legs have been shown to vary significantly with walking speed [14]. Further verification is required to determine if its effect increases or decreases the measure. Stance factor is unaffected by walking speed because stance factor is intrinsically expressed as a ratio. Because SF is the ratio of the contact duration between the sham and experimental foot, the difference in walking speed between two different trials does not change the ratio. Further studies including gait velocity as a covariate are necessary when comparing these analysis methods.

Poor video quality and toe contracture eliminated many videos and animals from the study. Although toe contracture did not eliminate animals from being evaluated with IC, TOA, and SF, it did prevent evaluation via SFI. To compare every evaluation method with the same animals, there were only n = 5 animals per group. This small sample size reduced the impact of the present study. Further, certain tests had requirements that limited my ability to evaluate. Specifically, imbalance coupling required a minimum of four consecutive steps at a constant speed [7]. Animals with recently severed sciatic nerves frequently take just one or two steps before resting. For many animals, out of ten or more videos, only one instance of four consecutive steps could be captured. If the data from this set of steps was an outlier, it was impossible to check other data from that animal to determine if the outlier was caused by a larger pattern or a one-time abnormality. In certain cases, measurement was taken on the last step

before the animal came to a halt. This change in velocity could cause the animal's gait to differ slightly than when in continuous motion. When the animal is coming to a halt, it may not swing its leg as far. This change would result in a shorter stance duration, causing SF to decrease and IC to increase and skew the data compared to trials where the animal took 4 or more steps at a constant speed.

The low R-squared values of IC, TOA, and SF compared to time indicate that the time point in the study is not able to predict the evaluation method scores. Because the evaluation method scores indicated recovery level, it is impossible for the time point in this study to indicate how recovered the animal is. This low regression value is due to a high variation in evaluation method scores. This large variation could be because of the small sample size or velocity variability and does not exclude these evaluation methods from further studies with higher sample sizes.

Based on the results here, changes in protocol in future walking track evaluations could better reveal the ability of these evaluation methods to quantify functional recovery. Direct lighting and a higher speed camera will improve accuracy in measurements. Toe out angle accuracy would be improved by using dye to mark the midline of the animal. The most important change to the protocol to increase the impact of this study would be the incorporation of a treadmill in the walking track analysis. Because three out of the four evaluation methods are affected by speed variability, it is difficult to draw any firm conclusions about them when speed is not controlled. While this method may be more expensive, it is feasible. For example, Jacobs, et al., 2018 [15] uses an open source GAITOR Suite while Deumens, et al., 2007 [16] uses CatWalk gait analysis in their sciatic rat model. Lastly, if treadmills are not implemented, the study could be improved by motivating the animals to walk across the track without pause. Conducting testing prior to feeding time and placing food at the end of the track would also motivate the animals to walk across the track without pause. These additions will reduce the length of time required to get a video with usable data.

CONCLUSIONS

This study served to assess the potential of four techniques to quantify the functional return after nerve damage. Video walking track analysis allowed for a retrospective evaluation of the sciatic functional index, imbalance coupling, stance factor, and toe out angle. Imbalance coupling showed promising correlation with the current industry standard, the sciatic functional index. Future investigation with updated protocol is necessary to confirm the degree of correlation and to evaluate the potential for a new industry standard for evaluating nerve repair.

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REFERENCES

1. Silva, J. B., Marchese, G. M., Cauduro, C. G., & Debiasi, M. Nerve conduits for treating peripheral nerve injuries: A systematic literature review. *Hand Surgery and Rehabilitation*, **2017,** 36(2), 71–85.

2. Rodrigues, M. C. O., Antunes, A., Jr, R., Glover, L. E., Voltarelli, J., & Borlongan, C. V. Peripheral Nerve Repair with Cultured Schwann Cells: Getting Closer to the Clinics. *Scientific World Journal,* **2012,** 1-10.

3. Muheremu, A., & Ao, Q. Past, Present, and Future of Nerve Conduits in the Treatment of Peripheral Nerve Injury. *BioMed Research International,* **2015,** 1-6.

4. Li, X., Wang, W., & Wei, G. Immunophilin FK506 loaded in chitosan guide promotes peripheral nerve regeneration. *Biotechnol Lett,* **2010,** (203),1333–1337.

5. Patel, M., Vandevord, P. J., Matthew, H., Wu, B. I. N., …Wooley, P. H. Video-Gait Analysis of Functional Recovery of Nerve Repaired with Chitosan Nerve Guides. *Tissue Engineering,* **2006,** (12), 3189-3199.

6. Meek, M. F., & Van Der Werff, J. F. A. Biodegradable p(DLLA-ε-CL) Nerve Guides Versus Autologous Nerve Grafts: Electromyographic and Video Analysis. *Muscle & Nerve,* **2001,** 753–759.

7. Bozkurt, A., Scheffel, J., Brook, G. A., Joosten, E. A., Suschek, C. V, Dey, D. M. O., & Deumens, R. Aspects of static and dynamic motor function in peripheral nerve regeneration: SSI and CatWalk gait analysis. *Behavioural Brain Research,* **2011,** 219 (1), 55–62.

8. Lee, J., Giusti, G., Wang, H., Friedrich, P. F., Bishop, A. T., & Shin, A. Y. Functional Evaluation in the Rat Sciatic Nerve Defect Model: A Comparison of the Sciatic Functional Index, Ankle Angles, and Isometric Tetanic Force. *Plastic and Reconstructive Surgery,* **2013,** (132), 1173–1180.

9. Wood, M. D., Kemp, S. W. P., Weber, C., Borschel, G. H., & Gordon, T. Annals of Anatomy Outcome measures of peripheral nerve regeneration. *Annals of Anatomy*, **2011,** 193(4), 321–333.

10. Varejao, A. S. P., Melo-pinto, P., Meek, M. F., Filipe, V. M., & Bulas-cruz, J. Methods for the experimental functional assessment of rat sciatic nerve regeneration, *Neurological Research,* **2004,** (26), 186-194.

11. Walker, J. L., Evans, J. M., Meade, P., Resig, P., & Sisken, B. F. Gait-stance duration as a measure of injury and recovery in the rat sciatic nerve model, *Journal of Neuroscience Methods*, **1994,** *52*, 47–52.

12. McKay Cavanaugh, Elena Silantyeva, Galina Pylypiv Koh, Elham Malekzadeh, William Lanzinger, Rebecca Willits, Matthew Becker. "RGD-modified nanofibers enhance functional outcomes in rats after sciatic nerve injury" In submission, **2019.**

13. Bain, J.R.M., S. E. & Hunter, D. A. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. *Plastic and Reconstructive Surgery*, **1989,** (83), 129-38.

14. Serradj, N., & Jamon, M. The adaptation of limb kinematics to increasing walking speeds in freely moving mice. *Behavioural Brain Research,* **2009,** (201), 59–65.

15. Jacobs, B. Y., Lakes, E. H., Reiter, A. J., Lake, S. P., Ham, T. R., Leipzig, N. D., … & Allen, K. The Open Source GAITOR Suite for Rodent Gait Analysis. *Scientific Reports,* **2018,** (8), 1–14.

16. Ronald Deumens, Robby J.P. Jaken, Marco A.E. Marcus & Elbert A.J. Joosten, (2007). The CatWalk gait analysis in assessment of both dynamic and static gait changes after adult rat sciatic nerve resection. *Journal of Neuroscience Methods* (164), 120-130.