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Mimicking exercise *in vitro* – effects of myotube contractions and mechanical stretch on omics

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ABSTRACT

The number of studies using skeletal muscle (SkM) cell culture models to study exercise *in vitro* are rapidly expanding. Progressively, more comprehensive analysis methods, such as different omics approaches including transcriptomics, proteomics and metabolomics have been used to examine the intra- and extracellular molecular responses to exercise mimicking stimuli in cultured myotubes. Among other techniques, exercise-like electrical pulse stimulation (EL-EPS) and mechanical stretch of SkM cells are the two most commonly used methods to mimic exercise *in vitro*. In this mini-review we focus on these two approaches and their effects on the omics of myotubes and/or cell culture media. Furthermore, besides traditional two-dimensional (2D) methods, the use of three-dimensional (3D) SkM approaches are increasing in the field of *in vitro* exercise mimicry. Our aim with this mini-review is to provide the reader with an up-to-date overview of the 2D and 3D models and the use of omics approaches to study the molecular response to exercise *in vitro*.

INTRODUCTION

Exercise has many beneficial health effects, but the underlying mediators that occur within skeletal muscle (SkM) are poorly understood. *In vitro* models of exercise, such as exercise-like electrical pulse stimulation (EL-EPS) and mechanical stretch have been developed to enable exclusive examination of SkM myotubes and their intra- and extracellular responses to contractions and/or passive lengthening. In this mini-review, EL-EPS refers to electrically induced contractions of cultured myotubes, and mechanical stretch refers to external forces applied to cultured cells to enable lengthening and tension of myotubes with the aim of mimicking exercise-like responses. Overall, these methods reproduce many of the key physiological (e.g., hypertrophy) and molecular (e.g., transcriptional, translational, and metabolic) changes induced by exercise *in vivo* (see reviews (1, 2)). Based on the rapidly growing literature, EL-EPS and/or mechanical stretch of cultured myotubes seem to be effective tools for investigating exercise-mimicking responses within a well-controlled environment (1, 2). Additionally, other approaches, such as pharmacological compounds, have been used to mimic exercise *in vitro*. However, they are not within the scope of this mini-review (for review, see (3)), nor are the responses to exercise-like stimuli in myoblasts.

Exercise can be used to treat and prevent multiple diseases, and the effects may be partially mediated by SkM-derived signaling molecules. Indeed, SkM is considered a secretory organ able to produce and release signaling molecules such as proteins, peptides, metabolites, and nucleic acids that can be transported within e.g., extracellular vesicles (EVs) to target nearby and/or distant cells (4). Although the nomenclature of these signaling molecules may differ depending on the study focus, for clarity, various exercise-induced molecules derived from the contracting SkM myotubes are referred to as exerkines herein (4). Although very few exerkines solely originate from SkM (5), it is plausible that SkM significantly contributes to the circulating exerkine pool, given its´ large proportion of the whole-body mass and extensive vascularization.

Alongside the development of novel research methods, the number of identified exerkines has expanded (6). However, the function and target tissues of many of these exerkines is still unclear and warrant future investigation. Omics-methods have become popular approaches to examine exerkines and exercise-induced changes in muscle epigenetics, transcription, protein signaling/translation and metabolism, both *in vivo* (7)

and, as demonstrated in this mini-review, *in vitro*. Our aim here is to provide the reader with a comprehensive overview of the recent advances of 2D and 3D *in vitro* exercise models combined with various omics techniques. Firstly, we summarize *in vitro* exercise omics studies and their relevance to *in vivo* exercise omics studies. Secondly, we discuss the important considerations related to *in vitro* exercise studies before outlining future perspectives.

OMICS OF THE CONTRACTING OR MECHANICALLY STRETCHED MYOTUBES

2D *in vitro* exercise and epigenomics

Epigenetics has become a popular area of interest within molecular exercise physiology given that epigenetic modifications can determine messenger RNA (mRNA) expression, ultimately affecting the response to exercise. Specifically, acetylation and methylation of histones as well as DNA methylation have received considerable attention (see reviews (8, 9)). Despite previous research demonstrating the importance of histone modifications during myogenesis in cultured SkM cells (10, 11) and in response to the exercise *in vivo* (8), to our knowledge, no studies have examined the histone response to exercise mimicking stimuli in cultured myotubes. Many groups, including us (12, 13) have investigated DNA methylation response to exercise *in vivo* as well as in aged (14) and mechanically stretched cultured myotubes (discussed below (15)). However, no studies have yet investigated DNA methylation after EL-EPS in 2D/3D, nor after mechanical stretch in 2D. Therefore, future studies utilizing genome-wide ´omic´ approaches to investigate the epigenomic response to exercise-like stimuli in cultured myotubes (e.g., EL-EPS, mechanical stretch, or a combination of the two stimuli (16)) is required.

2D *in vitro* exercise and transcriptomics

Several factors including reactive oxygen species (ROS) (17), contraction frequency (Hz) (17, 18), adrenaline (19), lack of long non-coding RNA taurine-upregulated gene 1 (*Tug1*) (20) and media glucose content (21) all regulate the transcriptome after EL-EPS in C2C12 myotubes (Table 1). Regarding the exercise-like stimuli in cultured myotubes, the transcriptional response has traditionally been analyzed via quantitative polymerase chain reaction (qPCR) and microarrays. These methods have demonstrated that EL-EPS upregulated the expression of exerkines, many of which are related to inflammatory and/or cytokine signaling (22–25). However, RNA sequencing (RNA-seq) has expanded the knowledge of the contraction-responsive genes in myotubes as discussed below.

Of the individual genes, nuclear receptor subfamily 4 group A member 3 (*NR4A3*) has been shown to be one of the most exercise- and inactivity-responsive genes in SkM across all *in vivo* acute/chronic and aerobic/resistance exercise studies analyzed (26). *In*

vitro, *NR3A3* mRNA expression increased after EL-EPS in primary human (26) and C2C12 (20) myotubes. Moreover, *NR4A3* silencing abolished contraction-induced glucose uptake and altered mRNA levels of many other exercise- and inactivity-responsive genes (21). Overall, this suggests that *NR4A3* has a critical role in SkM metabolism and adaptation to changes in physical activity (26). In addition to *NR4A3*, amphiregulin (*Areg*) has been shown to be upregulated after EL-EPS in C2C12 myotubes and in SkM of exercised mice (27) suggesting its´ role as a novel exerkine.

Two recent studies examined the transcriptome after EL-EPS in primary human myotubes (28, 29). Genes related to pro-inflammatory pathways were upregulated in myotubes originating from females with polycystic ovary syndrome relative to healthy controls (28). Although EL-EPS responses may be affected by donor characteristics (1, 3), a recent study suggests that age, sex, and EL-EPS parameters may not have substantial transcriptomic and proteomic effects (29).

Based on RNA-seq, we (21) and others (19, 29) observed that processes related to cytokine and inflammatory pathways were upregulated after EL-EPS in C2C12 myotubes. Local inflammation plays a role in SkM adaptation to exercise (30). After acute aerobic exercise, these effects might be partly mediated by monocyte attractants, such as cytokines from the cytokine families CXC and CC (26). Indeed, upregulation of *Cxcl1*, *Cxcl5* (CXC family) and *Ccl2* (CC family) in response to EL-EPS has been reported using transcriptomic (19, 21, 29) and targeted methods (23–25). These specific cytokines have been associated with exercise-induced macrophage infiltration into SkM to promote tissue regeneration and/or hypertrophy *in vivo* (5, 24, 31).

Although high-frequency EL-EPS (≥ 30 Hz (1)) induces greater transcriptome-wide changes than low-frequency EL-EPS (≤ 5 Hz (1)) in C2C12 myotubes (17, 18), the time effect cannot be ruled out. Indeed, these studies have compared the effects of low- and high-frequency EL-EPS on myotube transcriptome only after short-term (≤ 8h (1)) stimulation. In contrast, we (21) and others (19, 29) have reported that long-term (≥ 24h (1)) low-frequency EL-EPS is also sufficient to modify the C2C12 myotube transcriptome. Notably, based on pathway analysis, the processes identified after short- and long-term EL-EPS seem to differ. More specifically, while short-term stimulation promotes mitochondrial adaptations (18) as well as mitogen-activated protein kinase (MAPK), p53, glutathione and pentose phosphate pathway upregulation (17), long-term stimulation targets mostly contractility and cytokine and/or inflammatory processes (19,

21). Similar to the changes observed after exercise *in vivo* (26, 32), the intensity (for *in vitro* stimulation frequency and voltage) and stimulation duration seem to have an effect on the transcriptome of the contracting myotubes. However, due to the variability in EL-EPS parameters employed within the literature and such few studies conducted, a direct comparison of the results obtained across the different EL-EPS protocols is difficult. Future research is warranted to better understand the extent to which EL-EPS parameters affect the gene transcription in myotubes and the relevance for investigating the transcriptomic response to exercise *in vitro*. The use of primary human myotubes from different donors will allow one to decipher the inter-individual effects of exercise *in vitro*.

2D *in vitro* exercise and proteomics and secretome

The SkM proteome and secretome have been examined after acute exercise and long-term training *in vivo* (7, 33). Proteomics focuses mainly on tissue proteins, while secretome, a subfield of proteomics, examines secreted signaling factors (7). The secretome is typically analyzed via assessment of proteins, peptides, cytokines as well as EVs and their cargo (7). Proteins have been extensively studied after exercise *in vivo* (5) and EL-EPS *in vitro* (25, 34), while EV responses are restricted to exercise *in vivo* (35).

To date, there are a number of *in vitro* studies analyzing the proteome (36, 37), secretome (38) or both (29, 39) in response to EL-EPS in myotubes (Table 1). These studies reported increased intracellular abundance of proteins related to ATP production and that the sarcomeric Z-disc was one of the key targets of phosphorylation in contracting C2C12 myotubes (36). Additionally, phosphoproteomics identified filamin A-interacting protein 1 (FILIP1) in murine C2 cells as an important regulator of filamin homeostasis, which has been associated with the development of SkM cells (37). Recently, EL-EPS increased the content of cytoskeletal proteins in primary human myotubes derived from young male donors (40).

Secretome analysis of contracting primary human myotube media and human plasma after EL-EPS and acute endurance exercise and sprint intervals, respectively, identified growth differentiation factor 15 (GDF15) as a novel exerkine targeting adipose tissue (38). Another exerkine, thymosin β4, was identified from the media of the contracting C2C12 myotubes after EL-EPS as well as from the circulation after *in vivo* exercise irrespective of the exercise mode (39). Furthermore, CXC motif chemokines (e.g., CXCL1, CXCL2, CXCL5 and CXCL8) and other exerkines were increased in the

media of primary human myotubes after EL-EPS in comparison to non-stimulated controls (29). Together, these proteomic findings agree with the transcriptomic results discussed above showing that EL-EPS targets specific contractile machinery within the myotubes and that myotubes release similar exerkines to contracting SkM *in vivo*.

During the last few years, EVs have gained a lot of interest as potential mediators of exercise adaptation (41). The EVs, including exosomes, microvesicles, and apoptotic bodies that originate from all cell types (35) can be found from all biofluids and cell culture media (42). Therefore, *in vitro* exercise models could be feasible tools to study the contraction-induced EV release and EV cargo exclusively from the SkM myotubes. The EVs can transport different types of cargo, such as proteins, nucleic acids, and lipids (35). Myotube-derived EVs and their content in response to EL-EPS or mechanical stretch have remained an understudied topic. However, a recent study reported that EL-EPS had no effect on EV size distribution, protein yield or surface marker abundance in C2C12 myotubes (43). MicroRNAs (miRNAs) are common cargo of EVs, and they are known to modify gene expression in response to exercise *in vivo* (35). The most characterized SkM-derived and exercise-responsive miRNAs include miR-1, miR-206, miR-133a and miR-499 (35). We have analyzed the C2C12 cell-derived EVs and their miRNA content after low-frequency EL-EPS (21). We observed, in agreement with the *in vivo* studies (35), that packing of miR-1-3p, and possibly miR-133a-3p, into EVs was enhanced in response to myotube contractions (21). Taken together, as myotube derived EVs are currently an understudied topic, more studies are needed to better understand if *in vitro* models resemble this aspect of *in vivo* exercise.

2D *in vitro* exercise mimetics and metabolomics

Although the number of studies analyzing the metabolome after exercise *in vivo* is high (see reviews (7, 44, 45)), less is known about the effects of EL-EPS on myotube and media metabolome (17, 46, 47) (Table 1). We analyzed the metabolome of C2C12 myotubes and their culture media after 24-hour of low-frequency EL-EPS using nuclear magnetic resonance (NMR)-based approach (47). Others have used short-term low- and/or high-frequency EL-EPS protocol to examine C2C12 myotube metabolome using mass spectrometry (MS)-based approach (17, 46). We (47) and others (46) also compared the effects of media glucose content on the contraction-induced responses in the myotube and/or media metabolome. The difference between the NMR and MS approaches is that

NMR identifies highly abundant molecules very quantitatively, while MS enables identification of less abundant molecules, albeit less quantitatively. Thus, as the metabolites identified by these methods differ in number and composition, comparison between these above-described studies is introductory. This clearly highlights the need for further *in vitro* EL-EPS metabolomics studies comparing and even combining these two methods.

In line with the *in vivo* observations (44, 45), we demonstrated that EL-EPS affected intermediates and amino acids associated with energy metabolism in both myotubes and their media (47). Moreover, higher glucose availability augmented many of the intra- and extracellular metabolomic changes and under low glucose conditions, glucose was almost completely depleted from the cells and media (47). Others reported that glucose availability of the contracting C2C12 myotubes also regulated the intracellular content of adenine nucleotides and adenosine (46). More precisely, in glucose-depleted conditions, myotube contractions increased AMP content and attenuated ATP regeneration in comparison to high glucose condition (46). Further related to energy metabolism, Hoshino and colleagues showed that high-, but not low-frequency EL-EPS, affected metabolites related to carbohydrate metabolism (17). Similar to our findings (47) this research group observed that the C2C12 myotubes increase lactate production (17), thus suggesting that these glycolytic cells (48) rely heavily on glycolysis, especially during high-frequency EL-EPS.

Additionally, the volume or total number of contractions seem to be important. Low-frequency EL-EPS had only minor effects on the myotube metabolome after a short-term stimulation (17), while long-term stimulation significantly affected both the myotube and the media metabolome and small molecule content (47). Notably, we also analyzed the cell-free media metabolome to robustly estimate EL-EPS-induced metabolite uptake (e.g., glucose and serine) and release (e.g., lactate and acetate). However, further studies using isotope tracers are needed to examine dynamic metabolite turnover (i.e., fluxomics).

3D *in vitro* exercise mimetics and omics

In addition to traditional 2D cultures, 3D approaches are used to better mimic native cell-cell and cell-extracellular matrix (ECM) interactions within tissues. These 3D cultures combine biorelevant scaffold material and individual or combined cell types. In contrast

to 2D models, there are a limited number of omics studies analyzing the effects of EL-EPS or mechanical stretch in 3D muscle (49) (Table 1). To the best of our knowledge, there are no studies on 3D bioengineered SkM epigenetics, transcriptomics, or proteomics. However, contraction-induced changes in the metabolome of the tissue-engineered human SkM myotubes have been studied after low- and high-frequency EL-EPS (50). The results showed that 3D EL-EPS promoted glycolytic and fatty acid metabolic fluxes in primary human myotubes (50). Additionally, with increasing frequency, there was a greater abundance of different acylcarnitines, thus supporting the increased fatty acid metabolism observed after EL-EPS (50). *In vivo*, different acylcarnitine types have been shown to increase in blood after exercise (44). Similar to *in vivo* findings (51) and our observations after EL-EPS in 2D (47), the abundance of intracellular amino acids, such as branched chain amino acids (BCAAs), were also increased after EL-EPS in 3D muscle (50), thus suggesting amino acid recycling in response to exercise-like stimulation *in vitro*.

To our knowledge, there are currently no studies that have undertaken transcriptomic analysis after mechanical stretch in 3D SkM. However, we mechanically stretched C2C12 cells and assessed mRNA expression of 37 genes previously altered across the human transcriptome (12) and epigenome (13) after resistance exercise (RE) (15). Interestingly, mRNA expression of 86% (32/37 genes) and 95% (35/37 genes) genes analyzed *in vitro* were similar when compared to RE in human and rodent SkM, respectively (15, 52). Overall, this suggests that mechanical stretch of cultured myotubes alone (i.e., in absence of concentric shortening contraction) is sufficient for inducing comparable mRNA expression responses to RE *in vivo*. Nonetheless, future *in vitro* studies should consider transcriptomic analysis after mechanical stretch to identify novel mechano-sensitive genes and to directly compare the results with the *in vivo* transcriptome after RE. Moreover, unlike mRNA expression, targeted DNA methylation changes across the majority of genes analyzed *in vitro* did not mimic the epigenetic response to acute-RE in humans (15) warranting further studies using omics approaches with and without EL-EPS.

CONCLUSIONS AND FUTURE DIRECTIONS

Advances in cell culture models and analytical methods has opened new avenues within the field of exercise physiology. As the models and methods improve (e.g., become more affordable and user friendly), more scientists can utilize these approaches to further expand the knowledge in the field. At the same time, the modern world encounters problems due to sedentary lifestyle. To overcome this deleterious metabolic condition, more knowledge of the molecular mechanisms of physical activity and inactivity is needed. Omics analyses of SkM after exercise *in vivo* and exercise-like stimuli *in vitro* provide a new holistic perspective of the molecular mechanisms of exercise as each have their pros and cons (Figure 1). To sum up, mostly *in vitro* models and their omics-based analyses corroborate with findings observed after exercise *in vivo*. Nevertheless, novel findings have been obtained from *in vitro* studies, such as the discovery of putative exerkines (e.g., amphiregulin and GDF15), are worth investigating further.

The future of *in vitro* exercise models looks promising. The development of *in vitro* exercise systems using bio-printing or 2D/3D organoid/tissue-on-a-chip models could provide more advanced models for studying, for example, intercellular communication. Indeed, a recent study has developed a tissue-on-a-chip system to recapitulate *in vivo* metabolism (53). The 3D models also allow studies on myotube-ECM interactions, as the cells produce ECM components to support the structures.

It is also important to note the limitations of *in vitro* exercise models. Human and rodent muscle cell lines differ in their genetic properties, which may affect their responses to exercise-like stimulation (54). Importantly, regardless of the cell line used, myotube contractions are not visible immediately after the electrical current is applied to the media. Thus, if the stimulation time is shorter than the time to detect visible contractions (i.e., contractile components are not yet fully functional), changes in the studied parameters can possibly be caused, for example, by Ca^{2+} flux rather than contractions *per se*. Additionally, nutrition is a key regulator of metabolism *in vivo*, but this aspect has remained overlooked and understudied within *in vitro* exercise studies. Indeed, media composition can affect myotube metabolome, epigenome, transcriptome, proteome and even extraction of the EVs. Therefore, it is important that the media composition (including serum used (55)) is carefully considered and well-reported in future studies. Lastly, a major limitation of the current *in vitro* exercise models is the inability to apply

stimuli to induce active lengthening (i.e., eccentric contractions) of myotubes. Development of such approach should be one of the main aims of future studies.

To summarize, there is a clear need for more physiologically relevant *in vitro* exercise mimicking models. The importance of SkM and exercise research in the world that faces multiple issues due to passive lifestyle needs better understanding of molecules that could have beneficial systemic effects. Thus, combination of omics approaches could provide even broader overview of all the health benefits of exercise.

AUTHOR CONTRIBUTIONS

J.H.L., D.C.T., and L.Y-O., drafted the manuscript, J.H.L. and L.Y-O. prepared the figures and tables. A.P.S., R.K., S.P. and J.J.H., critically reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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Owing space limitation, we regret omitting citations to papers that have contributed to the *in vitro* exercise mimicking field. The figures were created with BioRender.com.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

FIGURE LEGENDS

FIGURE 1. Omics approaches have become popular in *in vivo* and *in vitro* exercise research. Both study models have their pros and cons that affect the outcomes and interpretation of the results. Despite the limitations of the *in vitro* exercise mimicry, it has been shown to have similar responses as *in vivo* exercise at the transcriptional,

translational, and metabolic levels. *NR4A3*, nuclear receptor subfamily 4 group A member 3; GDF15, growth differentiation factor 15; EVs, extracellular vesicles; miRNA, microRNA; BCAAs, branched chain amino acids.

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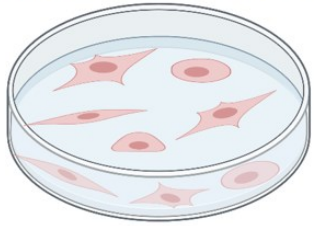
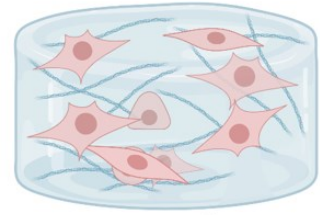
Approachies for *in vitro* exercise mimicry

Cells growing on a 2D dish

Cells growing on a 3D extracellular matrix (ECM)

Cells in 2D or 3D

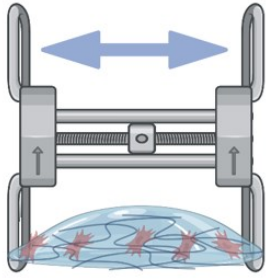


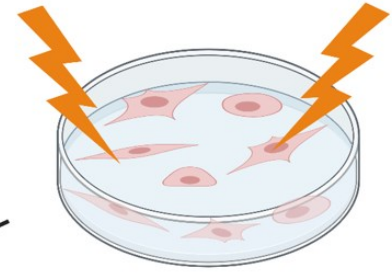


Mechanical stretching

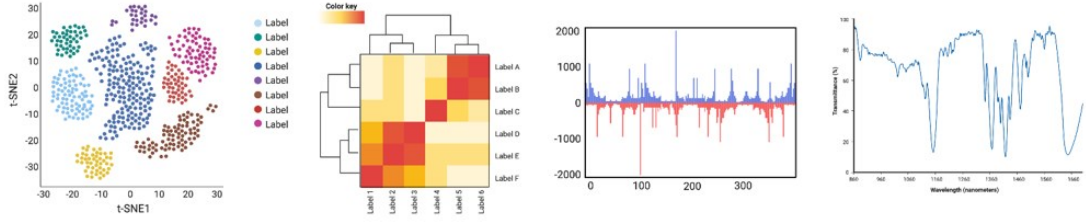
Exercise-like electrical pulse stimulation (EL-EPS)

Mechanical and/ or electrical exercise mimicing stimulation





omics analyses



In vivo

Pros

Intact neuromuscular and myotendinous junctions

Ability to study all types of exercise

Broadly reported pre- and post-exercise changes

Broader population range studied (age, sex, etc.)

Ability to observe systemic effects

Cons

Usually low cell specificity

Poor suitability for secretome studies

Time consuming and laborious

Participant dropouts, inability to monitor all eating, activity, etc.

Universal findings

Transcriptomics

Chemokines/cytokines ↑
e.g., CXC and CC families

Exercise-responsive genes ↑
e.g., *NR4A3*

Gene expression altered
e.g., by nutrients and ROS

Proteomics

Exerkine secretion ↑
e.g., GDF15, thymosin β4

EV release

Exercise-responsive miRNAs in EVs ↑
e.g., miR-1

Metabolomics

Energy metabolism ↑
e.g., glucose and lactate

Amino acid recycling ↑
e.g., BCAAs

Acylcarnitines ↑

In vitro

Pros

High cell spesificity, relatively easy and fast

Excellent suitability for secretome studies

Ability to control all parameters and conditions

Possibility to study direct intercellular crosstalk

Isolated test environment

Cons

Unideal cellular microenvironment

Murine cell lines more popular than primary human cell lines

Inability to study all types of exercise

Scarcely reported pre- and post-stimulation changes

TABLE 1. Overview of the different exercise-like electrical pulse stimulation (EL-EPS) parameters for investigation of *in vitro* exercise omics. The C2C12 and C2 cell lines are of murine origin, while HSkM are primary human skeletal muscle-derived cells.

Cell line	2D/3D	Duration	Stimulation parameters	Omics approach	Reference
C2C12	2D	2, 5, 15, 30 and 60 min. After 60 min + 1 h, 3 h or 6 h rest.	2 or 20 Hz, 50 V, 3 ms	Transcriptomics and metabolomics	(17)
	2D	3 h	Twitch: 2 Hz, 13 V, 2 ms Tetanic: 66 Hz, 13 V, 2 ms (5 s off, 5 s on)	Transcriptomics	(18)
	2D	3 h	66 Hz, 13 V, 2 ms, 5 s on and 5 s off	Transcriptomics	(20)
	2D	3 h	1 Hz, 30 V, 2 ms	Metabolomics	(46)
	2D	16 - 24 h	0.5 Hz, 10 - 12 V, 4 ms	Phosphoproteomics	(36)
	2D	24 h	1 or 10 pulses at 1 Hz, 20 V, 5 ms	Transcriptomics	(19)
	2D	24 h	1 Hz, 12 V, 2 ms	Transcriptomics	(21)
	2D	24 h + 1h rest	1 Hz, 11.5 V, 1 ms pulse stimulus of 2 ms duration	Transcriptomics	(27)
	2D	24 h	1 Hz, 11.5 V, 2 ms	Proteomics	(39)
	2D	24 h	1 Hz, 12 V, 2 ms	Metabolomics	(47)
HSkM	2D	3 h or 24 h	3 h: 0.5 Hz, 10 V, 24 ms 24 h: 0.1 Hz, 10 V, 2 ms	Proteomics	(38)
	2D	6 h	1 Hz, 11.5 V, 2 ms	Transcriptomics	(28)
	2D	24 h or 48 h	24 h: 0.1 Hz, 10 V, 2 ms 48 h: 1 Hz, 30 V, 2 ms	Transcriptomics and proteomics	(29)
	3D	1 h + 7 h rest, 1 week	1 Hz, 70 mA, 2 ms or 0.5 s 10 Hz pulse train every 5 s, 70 mA, 2 ms	Metabolomics	(50)
C2	2D	4 h	0.05 Hz, 10 V, 4 ms	Phosphoproteomics	(37)