MED64 The most sensitive microelectrode array system for *in vitro* extracellular electrophysiology

MED64 Protocol

Electrophysiological assessment of cerebral organoids

ALPHA MED SCIENTIFIC

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1. Introduction

Cerebral organoids (COs) are an increasingly popular model system used by neuroscientists to answer questions about the unique properties of human brain development. COs are generated from primary or induced pluripotent stem cells (iPSCs) and can be aggregated through self-organization without any direction or inductive signals (whole brain organoids) or in the presence of differentiation cues to form explicit regions of the human brain (midbrain, dorsal forebrain). Some studies have even generated different regions of the human brain and fused them together to create brain "assembloids". Patient-specific COs have been generated by reprogramming somatic cells such as fibroblasts or peripheral blood mononuclear cells (PBMCs) to iPSCs and then promoting differentiation into neuroectoderm. As COs grow and mature, they self-organize to form discrete regions of the human brain, including the cerebral cortex.

However, few studies have explored the electrophysiological properties of COs and how these properties develop across time. Using the MED64 in vitro micro-electrode array, it is possible to record multiple electrophysiological properties of COs and observe the maturation of such electrical phenotypes as the COs develop and increase their cellular diversity. This system allows for simultaneous acquisition of extracellular recordings and field potentials from 64 individual electrodes with a high signal:noise ratio.

The goal of this application note is to describe how to measure and determine multiple electrophysiological properties of COs, acquire relevant data, and extract the data for presentation or publication. This material has been prepared by scientists with expertise in neuroscience and electrophysiology. A protocol for plating, culturing, and carrying out experiments on COs has been prepared based on the users' experience.

1-1. Acknowledgement

Alpha MED Scientific would like to thank the MED64 users that have shared their knowledge:
Dominic Julian, MS – Research Associate, Institute for Genomic Medicine, Nationwide Children's Hospital
Mark E. Hester, PhD – Principal Investigator, Institute for Genomic Medicine, Nationwide Children's Hospital
Gong Cheng, MD- Senior Application Scientist, Alpha MED Scientific

1-2. Disclaimer

This application note is a summary of information shared by MED64 users and is to be considered marketing material. These methods have been developed, tested, and verified in the course of projects published in peer-reviewed literature. However, Alpha MED Scientific does not guarantee that the information written in this document is correct and is free from all liabilities. Please refer to the scientific literature for further insight on these techniques, as well as the MED64 and Mobius manuals for detailed instructions on use of the MED64 System.

2. Pretreatment of the MED Probes and MEA Recording of COs

2-1. Material to be prepared

Items	Supplier	Cat. No	Adjustment, Storage
Chemicals for coating Probe			
Polyethyleneimine	Sigma	P3143	
Na2B4O7/10H2O	Sigma	S9640	
Ethanol (70%)	Various		
Laminin	ThermoFisher	23017-015	Store at -80°C
Chemicals for creating Cerebral Organoid Growth and Differentiation Medium (for whole-brain organoid generation)			
BrainPhys Medium	Stem Cell Technologies	5790	Store at 4°C
N2 Supplement	ThermoFisher	17502-048	Store at -20°C
SM1 Supplement (containing Vitamin A)	Stem Cell Technologies	5711	Store at -20°C
MEM-NEAA	ThermoFisher	11140-050	Store at -20°C
GlutaMAX Supplement	ThermoFisher	35050-061	Store at -20°C
Insulin	Sigma	I9278-5ML	Store at -20°C
Antibiotic-Antimycotic (100X)	ThermoFisher	15240-062	Store at -20°C
2-mercaptoethanol (1:100 dilution)	Merck	8057400005	Store at 4°C
Equipment			
MED Probe	Alpha MED Scientific	MED-P515A	
Pipette, 1mL pipette tips, Petri dishes, Razor Blades, KimWipes, water bath, clean bench, CO2 incubator, Centrifuge, Microscope			

Please note: While this application note can be generalized to multiple cerebral organoid generation methods, this protocol will refer to the whole-brain method of generating cerebral organoids, and thus the reagents for the growth media referred to here are those for whole-brain organoid generation. Please consult your protocol about which growth media is appropriate.

2-2. Pretreatment of the MED Probe

CAUTION:

Avoid contact with the electrodes in all following procedures as they are extremely fragile.

Pretreatment of the MED Probe is necessary for recording the extracellular field potentials of COs. Without pretreatment, the electrodes will be unable to pick up the necessary signals from the COs and will not provide reliable data. Improper coating techniques can lead to disrupted recordings, thus the probes should be handled and managed carefully. This section contains recommended treatment of Probes for accurately and reproducibly collecting recordings of extracellular field potentials of COs.

Sterilization

1. Rinse a new MED Probe with sterilized distilled water (SDW) at least three times. Rinse it with 70% ethanol several times (or immerse it in 70% ethanol for 15 minute), and then let it dry naturally on a clean bench. Higher-grade ethanol is recommended to avoid deposits of organic substances onto the MED Probe after drying.

2. Rinse the MED Probe with sterilized distilled water (SDW) at least three times, and then let it dry under ultraviolet irradiation for 15-30 min. Store and handle the MED Probe in a sterilized 90 mm petri dish.

Pre-coating with PEI

The surface for the new MED Probe is hydrophobic. Thus, pre-coating with PEI is important for enhancing the hydrophilicity of the MED Probe's surface. While pre-treatment with PEI is not typically required when re-using MED Probes, some MED Probes may need to be pre-treated again after several uses.

Note:

Pre-coating should begin approximately 1.5 hours before the recording of the organoids occurs.

1. Treat the surface of a MED Probe with 0.005% Polyethyleneimine (PEI) in 25 mM borate buffer (pH 8.4) for ten minutes at room temperature. (Make sure the electrodes are covered by the PEI.)
2. Aspirate the PEI and coat with laminin at a concentration of 2µg/ml.
3. Incubate the Probe at 37°C for at least one hour.
4. Remove Probe from incubator and aspirate out Laminin solution. Rinse and aspirate with 200uL of PBS twice, and then place 200uL of PBS in the well.
    - If Probe is ready to be used, return to the incubator.
    - If Probe is not ready to be used, seal the top of the probe and the petri dish with parafilm. Place in a 4°C fridge *.
        - *Try to use the probe sooner rather than later- there is a chance that the coating on the electrodes may become compromised if left stored for months without use.

Recipe -PEI solution-

<u>25 mM borate buffer</u>

Dissolve 25 mM Na2B4O7/10H2O (MW: 381.4, Sigma: S9640) in distilled water and adjust the pH to 8.4 with HCl. (500 ml borate buffer solution)

1) 4.768g Na2B4O7/10H2O in 450 ml distilled water.

2) Adjust pH to 8.4 with HCl.

3) Add distilled water to 500 ml.

<u>0.005% PEI (polyethyleneimine) solution</u>

Since a 50% PEI solution (Sigma: P3143) is so sticky, it is recommended to prepare a 1% stock solution first (1 ml 50% PEI to 49 ml 25mM borate buffer). This 1% stock solution is then diluted to (0.005% PEI) for final use.

- The PEI solution can be stored in refrigerator up to 1 month.

2-3. Recommended experimental environment

37°C is the recording temperature recommended for recording from COs as to mimic the incubation conditions that they are typically grown in (alongside with 5% CO_2) However, the set temperature can vary depending on samples and nature of your experiments. To maintain the desired temperature, one of two methods are recommended.

1) Use of CO_2 incubator

Place the MED Connector (MED-C03) inside a CO_2 incubator. Please note that incubators can introduce noise. Please refer to page 29-36 in the MED64 Handbook (vol1) to mitigate noise introduced by an incubator. Particularly, recordings can be compromised by noise introduced by the rapid temperature increases that occur as incubators power on. Wait until the desired temperature is stable before beginning acquisition. (It could take several hours depending on the incubator.)

2) Use of the MED Heated Connector

The MED Heated Connector (MED-CP04) heats the MED Probe chamber from the bottom. Below are recommendations for using the MED Heated Connector with a Microclimate (MED-CC06).

1. Do NOT place the MED Heated Connector in an environment where temperature changes frequently, for example in the proximity of an air conditioner or heater.

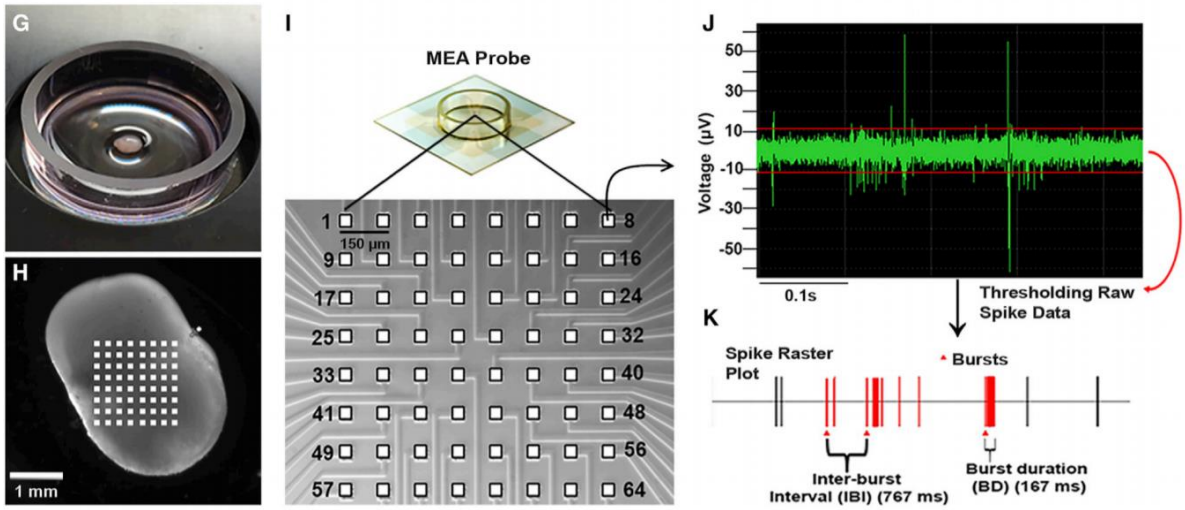
2. Cover the MED Probe to prevent ambient temperature fluctuation during acquisition.

3. It can take some time to reach the set-temperature. Power on the temperature controller connected to the MED Heated Connector at least 30 minutes before starting acquisition.

4. If/When the set-temperature is changed, wait for the temperature reported by the temperature controller to stabilize at the new temperature. It could take 5-30 minutes or more but is typically very fast.

2-4. Required Components

The MED Probe has 64 recording electrodes as well as 4 reference electrodes. The differences between the field potential acquired at the recording electrodes and the potential at the reference electrodes are measured by the MED64 System. Acquired signals are sent to the MED64 Head Amplifier through the MED Connector/MED Heated Connector. The raw signals are amplified by x10 by the Head Amplifier, and then amplified further by x20-217 and digitized with the MED64 Main Amplifier. We highly recommend reading the "Product manual" for each component, as well as the "MED64 Handbook", and "Mobius Tutorial" before using the MED64 System.



Above image from Stem Cell Reports, *Fair and Julian et. al, 2020.*

Required MED64 System Components

1) MED Probe
2) MED Connector (MED-C03) / MED Heated Connector (MED-CP04) *1
3) MED64 Head Amplifier (MED-A64HE1S)
4) MED64 Main Amplifier (MED-A64MD1)
5) Acquisition PC
6) Mobius software (Mobius Spike Sorter or Mobius Spike Sorter with stim package)

*1. MED Heated Connector with a temperature controller.

1. Sanitize the MED (Heated) Connector by cleaning with kim-wipe soaked with ethanol. (Don't clean the contact pins in the Connector).

2. Mount the MED Probe containing the cortical organoid or dissociated cells onto the MED (Heated) Connector.

CAUTION:

Clean the terminals on the outer portion of the MED Probe with a Kimwipe soaked in ethanol before mounting the MED Probe. Salt sediments can damage the contact pins on the MED (Heated) Connector.

3. Cover the MED Probe with a 35 mm petri dish or Perfusion Cap.

2-5. Placement of Cerebral Organoids onto MEA Probes

Preparation

- Using sterile equipment, cut the tips off 1mL disposable pipette tips to a diameter of 5mm to ensure cerebral organoids at all stages of development can be pulled up into them.
- Ensure that MED Heated Connector has reached the desired temperature of 37°C.

1. Remove MED Probe from fridge. Place in 37°C incubator for at least 15 minutes. Remove and aspirate out the PBS. The Probe is now ready to be used.

2. Add approximately 150uL of COGDM (or other appropriate culture media depending on protocol) to the surface of the probe.
    - This minimal amount of media allows the cerebral organoid to maintain close contact with the microelectrodes and still allows for low noise levels. However, if noise levels still reach unacceptable levels, increments of 25uL of COGDM can be added to the probe so that noise levels can be further reduced.
3. Transfer organoids from culture to the center of the probe containing the 64-channel array using a 1mL pipette with a 1mL tip previously cut to a 5mm diameter opening.
4. Place the probe within the MED64 system and keep the MED Heated Connector consistent at 37°C. It may be necessary to make fine adjustments to the placement of the organoid on the array using a 200ul tip to move the organoid.
5. Use the Mobius software to begin data collection.
6. Following data acquisition for up to 3 minutes (longer time intervals will result in evaporation of media from the chip, affecting the overall noise level), stop the recording and remove the cerebral organoid using another cut 1mL pipette tip to return it to its culture dish. Alternatively, one can perform longer recordings by maintaining the same volume of COGDM within the probe well. COs can then be returned to petri dishes with COGDM and placed in a tissue culture incubator for further growth and future data acquisitions.
7. Cortical organoid protocol developmental timeline, for more details, please see Stem Cell Reports, Fair and Julian et al, 2020.

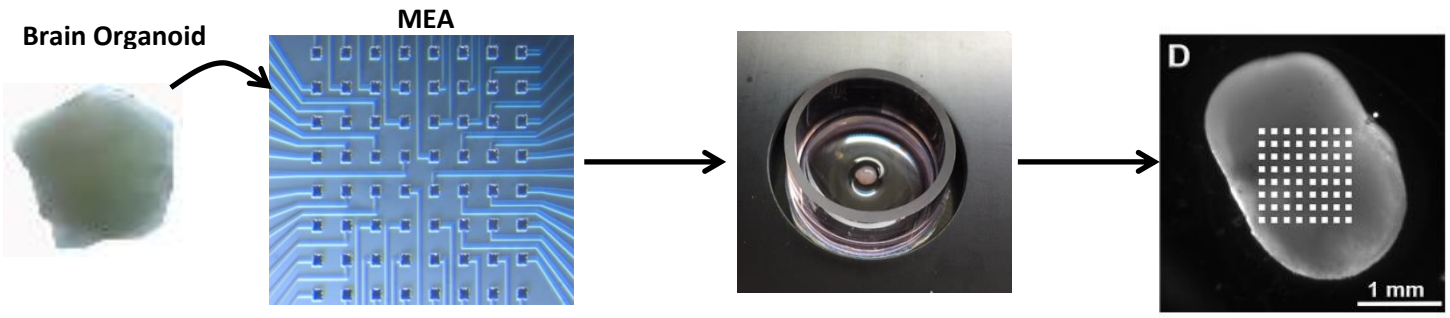


Fig. 1: Basic workflow of placing cerebral organoids onto probes for electrical analysis.

2-6. Cleaning the used MED Probes

The MED Probe's electrical characteristics are best during the first use. High quality signals can be recorded, and effective stimulation is possible with the MED64 System's MED Probes thanks to the electrode's characteristics, the lowest impedance in a commercially available microelectrode array. The electrode's impedance will increase with repeated use of the MED Probes due to damage in handling and/or residual cellular debris and coating materials. However, the MED Probes can be re-used if they are handled and cleaned very carefully. The following are protocols MED64 users recommend:

CAUTION:

ALWAYS avoid contact with the surface of the MED Probe to preserve the microelectrodes and insulation layer.

1. Aspirate or pipette off COGDM.
2. Rinse the MED Probe with PBS and aspirate. Repeat.
3. Add 500uL of PBS back into the MED Probe well.
4. Clean sides of MED Probe with 70% Isopropyl Alcohol to remove smudges or dirt.
5. Place parafilm around the probe, place in a petri dish and then place the dish in a refrigerator (4°C).

3. Data acquisition

According to Fair et. al, 2020, electrical activity in COs can be recorded using the MED64 by measuring extracellular field potentials. The authors highlight that day 34 of a whole brain organoid generation method is around the time that electrical activity can first appear within COs (using the whole brain method). After day 34, electrophysiological activities mature and ultimately result in synchronized bursting activity indicative that neuronal networks are forming in organoids. The recommended environment for recording from COs is in the following section.

Available Mobius workflow templates

Data can be acquired immediately using the available Mobius workflow templates enumerated below (please refer to page 43, Chapter 3: Spike Sorter on the Mobius Tutorial):

1. Spontaneous_recording (Recording WITHOUT any real-time analysis).
2. Spike_recording (Recording with extracting spikes and their frequency analysis).
3. Spike_recording_filter (2 + filter).
4. Spike_recording_cluster (Recording with extracting, clustering spikes, and their frequency analysis).
5. Spike_recording_filter_cluster (4+filter).

Recommended acquisition settings

The following parameters are recommended to perform data acquisition and analysis of neuronal spikes:

Input Range (Maximum input signal level): 2.9 mV
Low cut frequency (High pass filter): 100 Hz
High cut frequency (Low pass filter): 10000 Hz

Acquisition bandwidth can be narrowed to further decrease the baseline noise level.

4. Data analysis

The MED64 can easily record extracellular field potentials from COs. The Mobius Spike Sorter package is available to extract and analyze electrical activity spikes as well as burst duration and interburst activity. Analyses as well as raw data can be exported for processing using other software packages.

4-1. Workflow templates available for analyzing neuronal spikes

The Mobius Spike Sorter package has 4 built-in analysis workflow templates available for analyzing neuronal spikes.

"Spike_frequency_analysis", "Spike_frequency_analysis_filter"

These workflow templates allow you to:

- Set thresholds.
- Extract spikes that go over the pre-determined thresholds.
- Counts spike frequencies for the extracted spikes.
- Graph the spike frequency chart.
- Export raw data
- Export waveform of extracted spikes and their time stamps.
- Export the spike frequency chart.

For the *"Spike_frequency_analysis_filter"* workflow template, all above analyses are performed on filtered data.

"Spike_sorting", "Spike_sorting_filter"

These workflow templates allow you to perform same analysis as above. However, the extracted spikes are clustered based on their waveform similarities. The spike frequency is computed independently for each cluster.

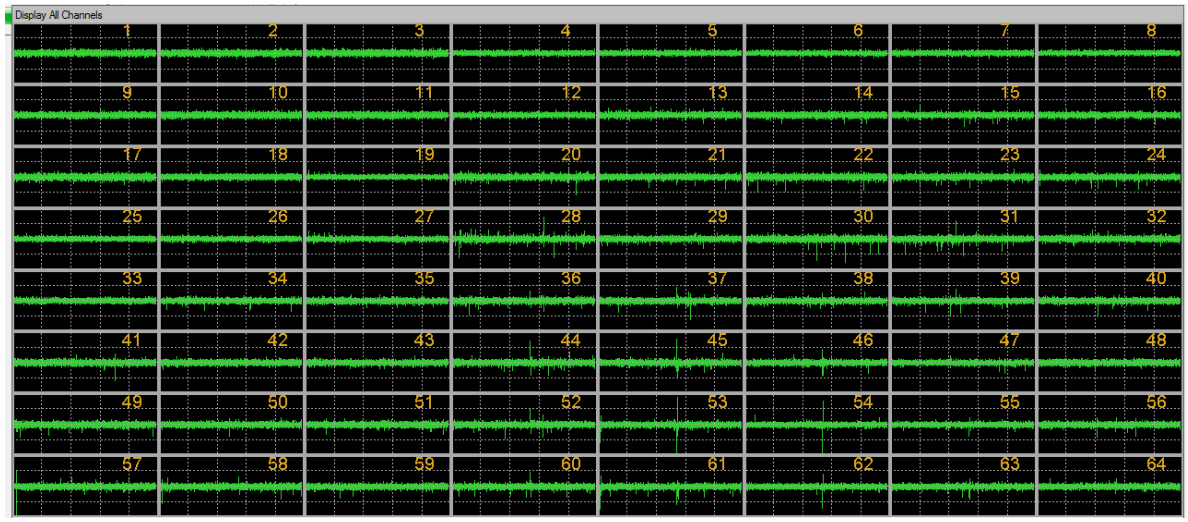
For the *"Spike_sorting_filter"* workflow template, all analyses are performed on filtered data.

4-2. Setting thresholds

All spike sorter workflow templates build in the [Extract Spikes Advanced] module, where thresholds are set by:

1. Typing numbers in the chart. (Top-left in the Figure 10)
2. Moving bars in the single channel display. (Top middle in the Figure 10)
3. Setting thresholds as SD (Standard Deviation) percentage. (Top right in the Figure 10)
4. Moving bars in the 64ch display. (Bottom in the Figure 10)

Spikes that go over the thresholds are extracted, and their frequencies analyzed.



Spike recordings and extraction for day 160 cerebral organoids with the [Extract Spikes Advanced] module.

4-3. Spike frequency analysis

Mobius Spike Sorter's built-in "Spike_frequency_analysis_(filter)" workflow templates can extract neuronal spikes and analyze their spike frequencies during recording and/or post-acquisition. Time course of spike frequencies are computed and graphed. Please refer to page 63 in the Mobius Tutorial for detailed instructions.

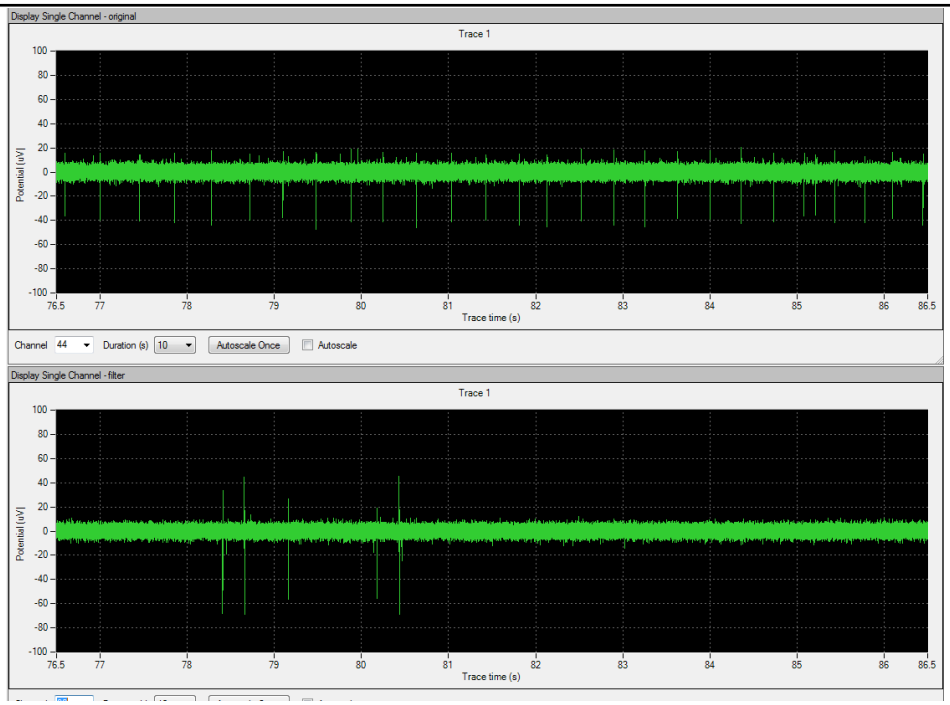
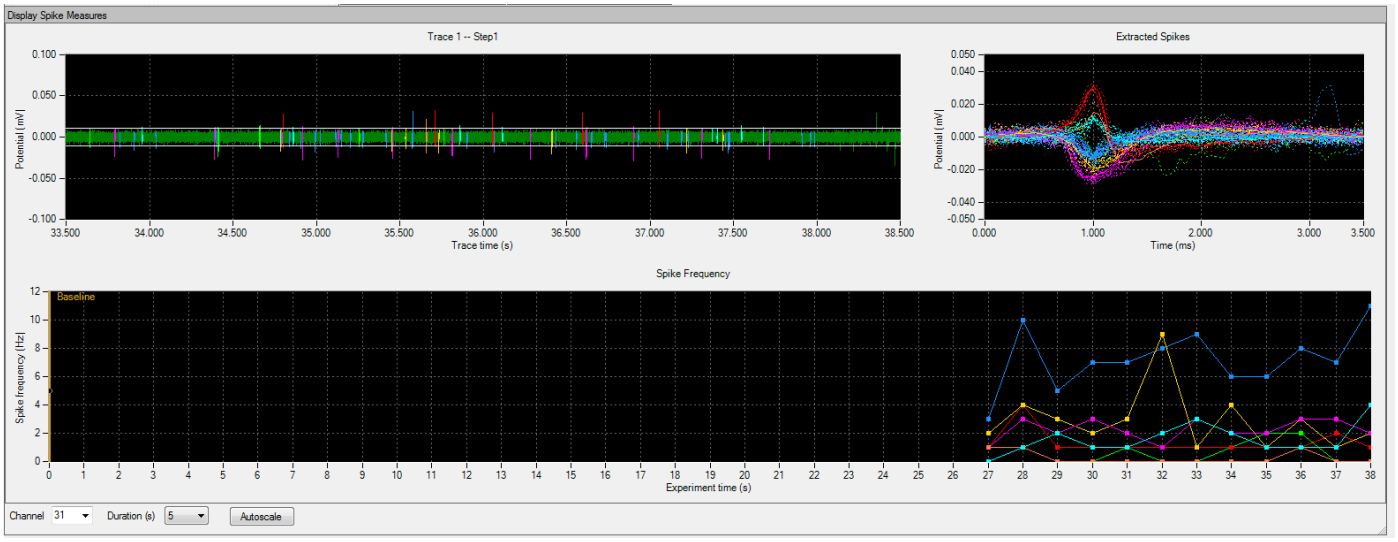


Figure 11. Spike frequency analysis using the *"Spike_frequency_analysis_filter"* workflow template. Spike frequencies for all extracted spikes are computed and graphed at each electrode.

4-4. Spike Sorting

The *"Spike_sorting_(filter)"* workflow templates extract neuronal spikes, sort them based on similarity of waveforms, and analyze their spike frequencies.



Spike sorting.

4-5. Exporting Data

Mobius can export data in user friendly formats for analysis with other software.

Data to be exported	Module	File type
Raw data	Export Raw Data	Binary / ASC II
Time stamp for extracted spikes	Save Spikes	ASCII
Waveform for extracted spikes	Save Spikes	ASCII
Values for spike frequencies	Save Spike Freqs	ASCII
Waveform for centroids	Cluster Spikes	ASCII

Data is exported as ASCII (or binary for Raw data) when:

1) The check-box for the module is checked, and
2) Data is replayed with the Green/Red button.

Channels for exporting are selected via the channel selector in the [Replay Raw Data File]. Traces and times to be exported are also selected at the trace/time counter in the [Replay Raw Data File].

Following export of data, raw data should be searched for spikes by using a threshold of 4.25x the SD of the raw signal. Spike data should then be imported into the **MED64 Mobius Offline** software (Version 1.4.5) where the following electrophysiologic metrics can be determined:

- Mean Spike Rate
- Local Field Potentials
- Number of Spikes
- Number of Bursts
- Spike Rate (Spikes/second)
- Interburst Interval
- Burst Duration
- # of Spikes within a Burst

A "**burst**" can be defined **as a simultaneous synchronous activity within a system** (Fair et al. 2020)

A "**spike**" is defined **as 4.25x the SD rate of the raw signal** (Fair et al. 2020)

An "**active**" **electrode** can be defined **as active by detecting at least 5 spikes/minute** (McConnell et al., 2012; Novellino et al., 2011; Fair et al. 2020).

Further parameters can be seen on the table on the following page.

Burst Detection Parameters	
maximum interval to start burst	300ms
maximum interval to end burst	301ms
minimum number of spikes in a burst*	10 spikes
minimum duration of a burst	50 ms
minimum interval between bursts	200ms
*except for the D34 time point containing 5 minimum number of spikes	
Network Burst Detection Parameters	
minimum percentage of active electrodes	25%
minimum threshold	1,100 spikes/second
bin size	100ms

Above table from Stem Cell Reports, *Fair and Julian et. al, 2020, Supplementary Information*

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