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

MED64 Protocol

Axol Human iPSC-derived Sensory Neuron Progenitor

ALPHA MED SCIENTIFIC

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Alpha MED Scientific Inc.
Saito Bio-Incubator 209, 7-7-15, Saito-asagi,
Ibaraki, Osaka 567-0085, Japan
E-mail: support@med64.com
Website: http://www.med64.com

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1. Introduction

Stem cell derived neurons can provide insights into mechanisms of the brain, behavior, and disease. Sensory neurons that transduce pain are crucial to protect an organism from potentially damaging external stimuli. Dorsal root ganglions (DRG) are pain-related neurons that have a variety of sensory receptors that are activated by chemical, thermal, and mechanical stimulation. Due to the intricate yet subtle activity of stem cell derived sensory neurons, a high fidelity assay is needed to measure and examine their activity. The MED64 is a high fidelity microelectrode array (MEA) platform that is engineered to detect a broad range of action potentials that stem cell derived sensory neurons produce.

The MED64's broad acquisition bandwidth combined with superior signal to noise ratio and carbon nanotube technology affords impeccable extraction of basic electrophysiological variables. The combination of precise extraction of electrophysiological activity with the ease of culturing cells directly onto the MED Probe (Multi Electrode Dish) makes the MED64 ideal for pharmacological, drug safety screening, and basic scientific applications. The 64 electrodes on the MED Probe have the lowest impedance of any micro-electrode array, making the MED64 ideal for acquiring data from spontaneously spiking neurons or neurons that fire in response to drug application. The MED64 also has high capacitance electrodes, enabling the MED64 to deliver high stim- ulating current, which is essential for evoked response studies.

The goal of this protocol is to describe how to set up experiments with dissociated sensory neuron cultures, 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 complete protocol for plating and culturing neuron cultures 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,:

Ikuro Suzuki,PhD - Tohoku Institute of Technology
Aoi Odawara,PhD - Tohoku Institute of Technology
Zoe Nilsson,PhD - Product manager, Axol Bioscience
Nick Clare,PhD - Application scientist, Axol Bioscience
Michael Trujillo,PhD - Global product manager, Alpha MED Scientific

1-2. Disclaimer

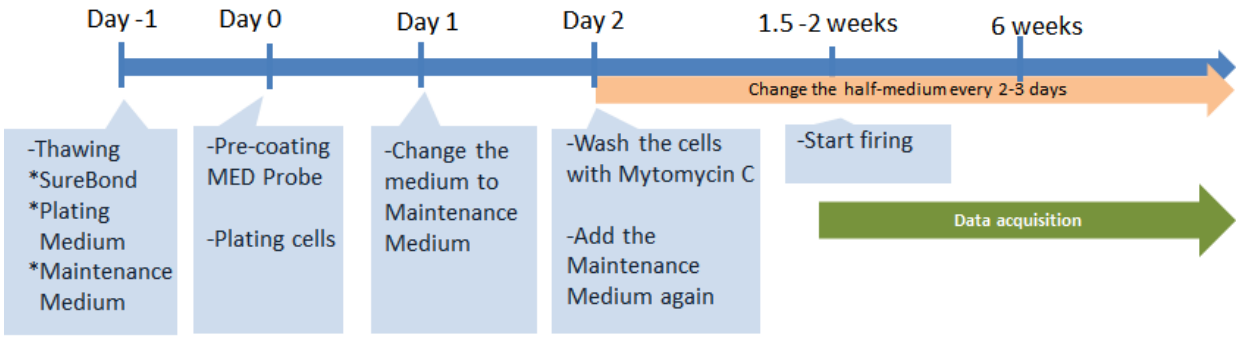
This application note is a summary of information shared by MED64 users and is to be considered marketing material. These methods have been developed, tested, and verified in the course of projects published in peer-reviewed literature. However, Alpha MED Scientific does not guarantee that the information 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 plating sensory neurons

2-1. Material to be prepared

Items	Supplifer	Cat. No	Note
Cell			
Human iPSC-Derived Sensory Neuron Progenitor	Axol	ax0055	>500,000 cells / vial
Reagents for coating			
SureBond+ReadySet	Axol	ax0041	
(SureBond)			Store at -80 °C
(ReadySet)			Store at 4 °C
Reagents for cell plating and cultureing			
Neural Plating-XF medium	Axol	ax0033	Store at -80°C
Sensory Neuron Maintentance Medium	Axol	ax0060	Store at -80°C
Mitomycin C	Sigma	M4287	Store at -20°C
Glial-Derived Neurotrophic Factor (GDNF)	Peprotech	450-10	Store at -20°C
Nerve Growth Factor (NGF)	Peprotech	450-01	Store at -20°C
Brain-Derived Neurotrophic Factor (BDNF)	Peprotech	450-02	Store at -20°C
Neurotrophin-3 (NT-3)	Peprotech	450-03	Store at -20°C
Equipment			
MED Probe	Alpha MED Scientific	MED-R515A (or MED-P515A)	
Cloning ring	Iwaki	11-0162 (RING-05)	ID:3.4, OD:5, Height:10 (mm)
35mm culture dish	Various		
Conical tube (15, 50 ml)	Various		
Microtube (2.5ml)	Various		
Pipet, Petri dishes, kim wipes, waterbath, clean bench, CO2 incubator, Centrifuge, microscope			

2-2. Workflow



2-3. Preparation of Regents

- Please read product manual and protocol by Axol or other suppliers.

Neural Plating-XF Medium

- Upon receipt, store Neural Plating–XF Medium (Axol Cat# ax0033) at or below -80°C protected from light.
- When ready to use, thaw Neural Plating–XF Medium overnight at 4°C in the dark.
- Once thawed, Neural Plating–XF Medium must be used and cannot be refrozen.

Sensory Neuron Maintenance Medium

- Upon receipt, aliquot and store Sensory Neuron Maintenance Medium (Axol Cat# ax0060) at or below -80°C protected from light.
- When ready to use, thaw an aliquot of Sensory Neuron Maintenance Medium overnight at 4°C in the dark. (Thawing the Sensory Neuron Maintenance Medium can take longer than overnight.)
- Prepare Sensory Neuron Maintenance Medium by adding the following:

Growth Factor	Final Concentration
Glial-Derived Neurotrophic Factor (GNDF)	25 ng/mL
Nerve Growth Factor (NGF)	25 ng/mL
Brain-Derived Neurotrophic Factor (BDNF)	10 ng/mL
Neurotrophin-3 (NT-3)	10 ng/mL

- Growth factors should be added fresh each time an aliquot of Sensory Neuron Maintenance Medium is thawed.
- A thawed and supplemented aliquot of Sensory Neuron Maintenance Medium can be stored at 4°C for 1 week.

Mitomycin C

- Prepare a 0.5 mg/mL stock concentration of mitomycin C by solubilizing 2 mg in 4 mL of ddH2O. Make 50-100 µL aliquots of mitomycin C (0.5 mg/mL), protect from light and store in a dark box at 4°C. Stored at 4°C, mitomycin C is stable for up to 8 weeks.

Sensory Neuron Maintenance Medium containing Mitomycin C

- Prepare medium containing 2.5 µg/mL of mitomycin C by adding 100 µL of the 0.5 mg/mL stock of mitomycin C to 20 mL of Sensory Neuron Maintenance Medium.
- This medium should then be filter sterilized prior to use using a 0.22 µM filter.

2-4. Pretreatment of the MED Probe

CAUTION:

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

Pretreatment of the MED Probe is the most critical step to culture neurons onto the electrodes. The surface of a new MED Probe is hydrophobic. Therefore, hydrophilization of the MED Probe is necessary to enhance the adhesion of neurons to the electrodes. If cells do not adhere to the electrodes, you will not be able to record from them. It should be noted that some coating agents can affect neural activity, viability, degree of neurite outgrowth, extent of migration, and longevity. Improper coating techniques can cause large-scale clumping and/or the death of neurons even if all other cell culture steps are performed properly. The following section contains recommendations for appropriate pretreatment of the MED Probes.

Sterilizing the MED Probe

1. Rinse a new MED Probe with sterilized distilled water (SDW) at least three times. Immerse it in 70% ethanol for 15 minute). Higher-grade ethanol is recommended to avoid deposits of organic substances onto the MED Probe
2. Rinse the MED Probe with SDW at least three times and then let it dry naturally on a clean bench.
3. Place the MED Probe under ultraviolet irradiation for 15-30 min.
4. Store and handle the MED Probe in a sterilized 90 mm petri dish.

Pre-coating the MED Probe

- Thaw the Surebond (Axol Cat# ax0052) at 4 °C overnight the night before plating.

1. Add 950 uL of Readyset (250 uL / Cm2) into the MED Probe (MED-R515A or MED-P515A), and incubate at 37ºC for 45 minutes.
2. Remove the Readyset solution, and immediately rinse the Probe with sterilize water 4 times. Fill the Probe with sterilize water.
    - Pay attention to not let the Probe surface dry during this process.
3. Mix SureBond 120uL with D-PBS 6ml and pipette it.
4. Remove sterilized water from the Probe and immediately fill the Probe with 800 uL of SureBond solution (#3).
5. Incubate in an incubator (37 °C, 5%CO_2) for one hour.

2-5. Thawing and Preparing the sensory neurons

- Please read product manual and protocol by Axol or other suppliers.

1. Transfer the vial of Axol sensory neurons (Axol Cat# ax0055) from storage by transporting the vial covered in dry ice. Remove the vial from dry ice and transfer it to a 37ºC water bath.
2. Quickly thaw the vial of sensory neurons in a 37 °C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after 1-2 minutes.
    - Do NOT shake the vial during thawing.
3. Take the vial of sensory cells to a biological cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.
4. Using a p1000 pipette, transfer the cell suspension into a 15mL sterile conical tube. Gently wash the cryogenic vial with 1mL of warm Neural Plating-XF Medium and transfer this to the 15mL sterile conical tube.
5. Add 8 mL of Neural Plating-XF medium drop-wise to the cell suspension.
6. Centrifuge cells at 200 xg for 5 minutes at room temperature.
7. Aspirate and discard the supernatant carefully with a pipette.
8. Using a P1000 pipette, gently resuspend the cell pellet in 1 mL of Neural Plating-XF Medium (ax0033) until they are in a single cell suspension.
9. The ideal cell volume is 1,000,000 cell/ml.

2-6.Plating the sensory neurons onto the MED Probe

CAUTION:

Be careful NOT to touch the electrodes.

Preparing for cloning ring

The sensory neurons will be plated onto electrodes using a cloning ring. Prepare the cloning rings before plating.

1. Autoclave cloning ring and place them in a petri dish (Figure 1, Left).
2. Shake the petri dish. Select only cloning rings that do NOT move (Figure 1, Right).
    - Some cloning rings have uneven edge, which can cause cell suspension to leak. This step is necessary to select only the good cloning rings.

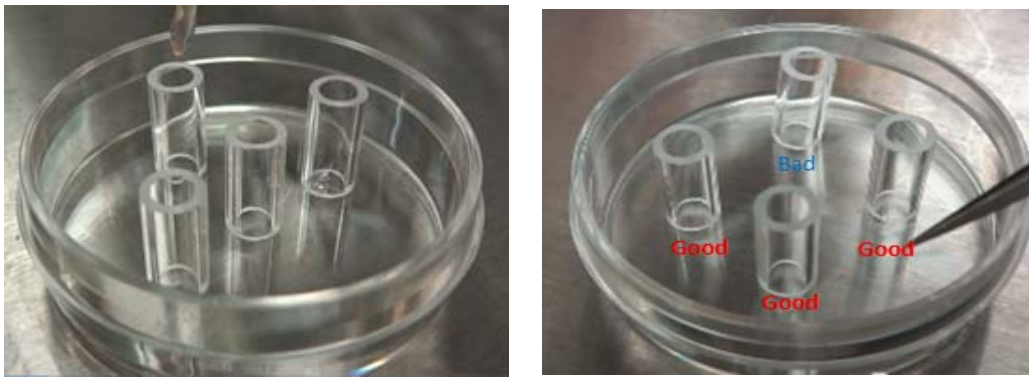


Figure1. Cloning ring placed in a petri dish (left). Shake it and select only cloning rings that do NOT move (right).

3. Moisten the inside of the cloning ring by pouring Neural Plating-XF Medium (Figure 2, left).
    - This process will help prevent the cell suspension from stacking in the middle of cloning ring (Figure 2, right).

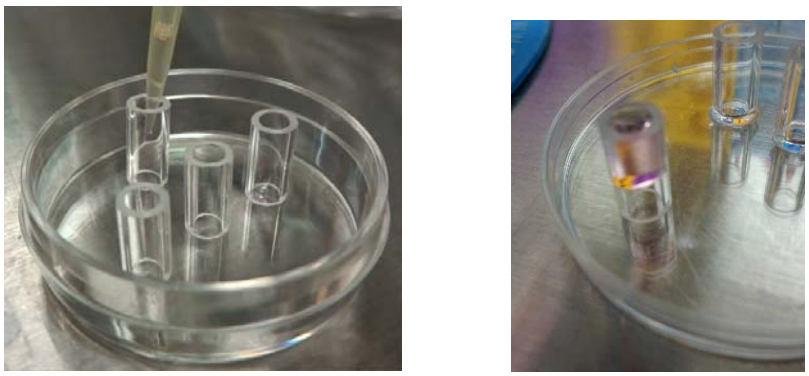


Figure 2. Moisten the inside of the cloning ring (left) and cell suspension stacking in the middle of cloning ring.

Plating cells onto electrodes using the cloning ring

1. Remove the SureBond from the MED Probe (Figure 3, left), and place a moistened cloning ring in the center of the MED Probe (around the electrodes) (Figure 3,right).

Figure 3. Removing the SureBond (left) and Placing cloning ring around the electrodes (right).

2. Flood **80 uL** cell suspension (containing around **80,000 cells**) into the cloning ring (Figure 4, left).
3. Add 1 mL of Neural Plating-XF Medium around the cloning ring (Figure 4, right).

Figure 4. Flood cell suspension into the cloning ring (left) and add Neural Plating-XF medium around the ring.

4. Incubate it at 37ºC, 5%Co_2 for one hour.
5. Remove the cloning ring gently (Figure 5).

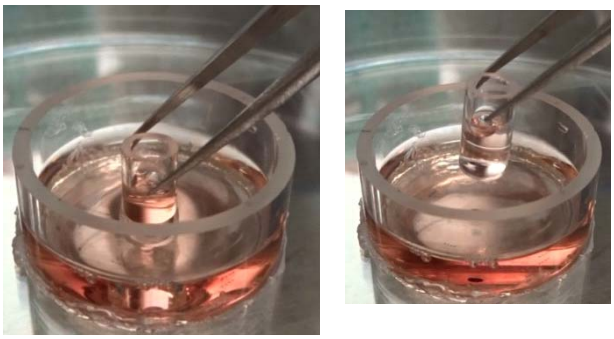


Figure 5. Removing the cloning ring.

On the following day (Day 1)

6. Replace the medium with 1 mL of fresh pre-warmed, 37ºC Sensory Neuron Maintenance Medium (ax0060) Supplemented with GDNFF (25 ng/ml), NGF(25 ng/ml), BDNF(10 ng/mL), NT-3(10 ng/mL).

On the second day after thawing (Day2)

7. Remove all the culture medium in the Probe, and replace with 1 mL of Sensory Neuron Maintenance Medium containing 2.5 ug/mL of mitomycin C.

8. Incubate the Probe for 2 hours at 37ºC, 5%CO_2.

9. Remove the Sensory Neuron Maintenance Medium containing 2.5 ug/mL of mitomycin C from the MED Probe, and gently wash the cells in the Probe once with pre-warmed 37ºC D-PBS (1x) (without calcium or magnesium).

10. Fill the MED Probe with 1 mL of 37ºC Sensory Neuron Maintenance Medium supplemented with GDNF (25 ng/mL), NGF (25 ng/mL), BDNF (10 ng/mL).

    - Mitomycin C treatment is not effective immediately. Non-neuronal cell death will not occur until 4-5 days after treatment. Full effects will be apparent after 7 days.

11. Replace half the volume of medium with fresh pre-warmed 37ºC Sensory Neuron Maintenance Medium supplemented with GDNG(25 ng/mL), NGF(25 ng/mL), BDNF (10 ng/mL), NT-3 (10 ng/ml) every 3-4 days.

12. Sensory neuron starts firing 1-2 weeks after plating, however you may want to perform your experiments in 6 weeks or later after synchronized firing is observed.

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.

Trypsin method

1. Fill a used MED Probe with 0.25% Trypsin, and incubate at 37°C for 1 hour.

2. Rinse the MED Probe with PBS 3 times.

3. Rinse the MED Probe with sterilized distilled water (SDW) at least 3 times.

4. Fill the MED Probe with SDW, and store the MED Probe in a 90 mm petri dish.

5. Store the petri dish in refrigerator.

3. Data acquisition

Cultured sensory neurons start firing in 1-2 weeks after plating, but initial activity can vary depending on culturing technique. Once the neurons begin firing spontaneously, activity can be recorded using the MED64, however you may want to perform your experiments in 6 weeks or later until synchronized firings are observed. The following section details the recommended environment for recording from sensory neurons.

3-1. Recommended experimental environment

37°C is the typical recording temperature recommended for recording from sensory neuron cultures. 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. 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.)

Figure 6. Experiment with the MED Connector inside a CO2 incubator.

2) Use of the MED ThermoConnector

The MED ThermoConnector (MED-CP04) heats the MED Probe chamber from the bottom. Below are recommendations for using the MED ThermoConnector.

1. Do NOT place the MED ThermoConnector 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 ThermoConnector's temprature controller 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.

3-2. Data acquisition

The MED Probe has 64 recording electrodes as well as 16(or 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 ThermoConnector. The raw signals are amplified by x10 by the Head Amplifier, and then amplified further and digitized with the MED64 Main Amplifier. We highly recommend reading the "Product manual" for each component, as well as "Mobius Tutorial" before using the MED64 System.

Required MED64-Basic System Components

1) MED Probe
2) MED Connector (MED-C03) / MED ThermoConnector (MED-CP04)
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. Sanitize the MED (Thermo)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 cells onto the MED (Thermo)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 (Thermo)Connector.

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

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 typically recommended to perform data acquisition and analysis of neuronal spikes:

Input Range (Maximum input signal level):	2.9 mV
Low cut freq (High pass filter) :	100 Hz
High cut freq (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 spikes from active neurons. The Mobius Spike Sorter package is available to extract and analyze neuronal spikes. 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.

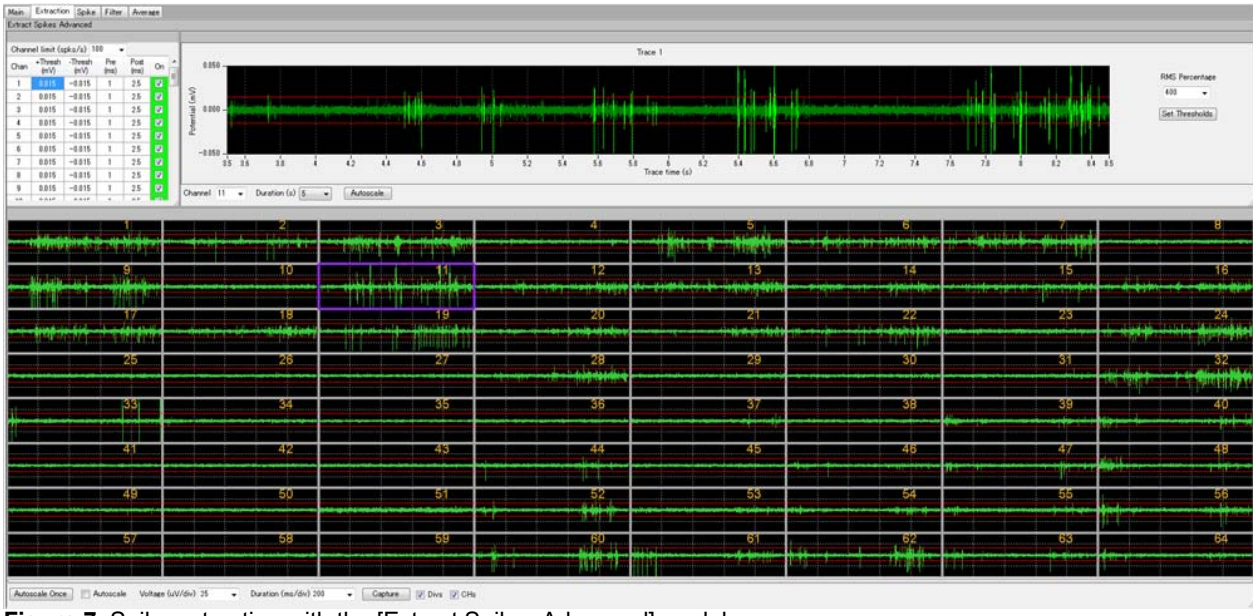


Figure 7. Spike extraction with the [Extract Spikes Advanced] module.

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.

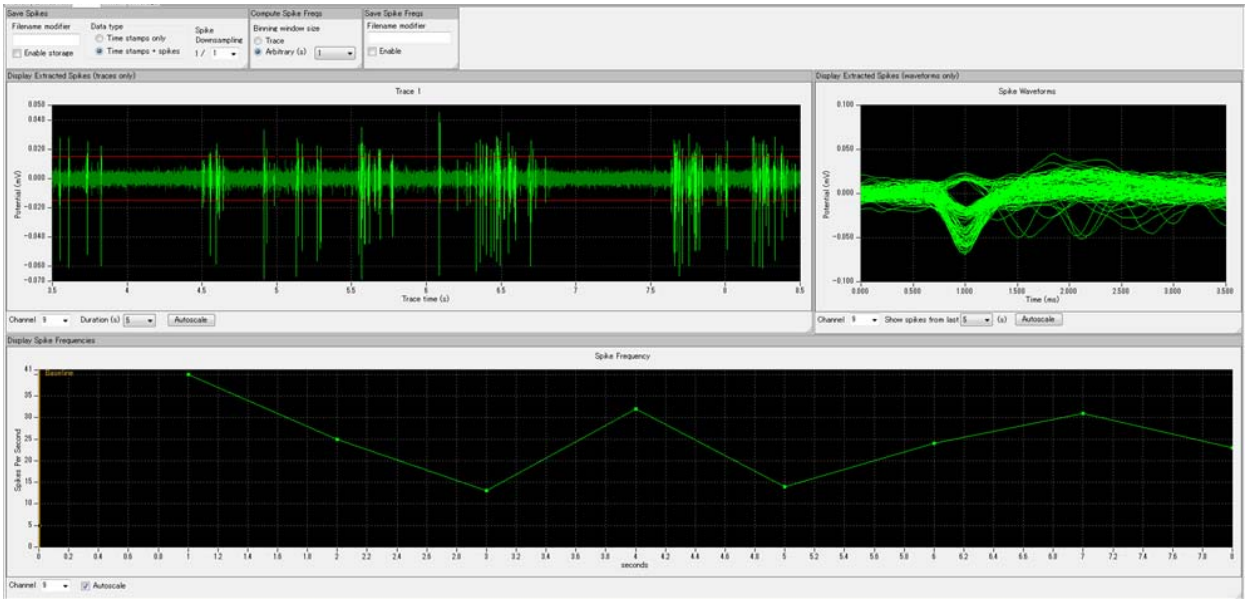


Figure 8. Spike frequency analysis using the *"Spike_frequency_analysis_filter"* workflow template. Spike frequencies for all extracted spikes are computed and graphed at each electrode.

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.

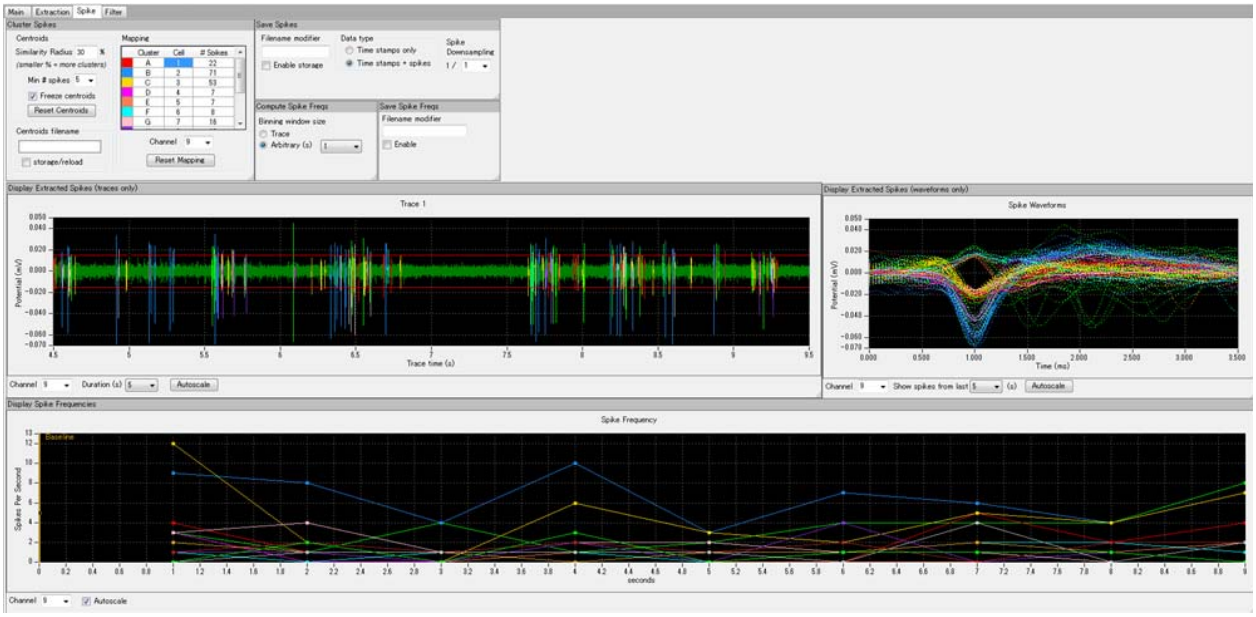


Figure 9. Spike sorting.

4-5. Exporting Data

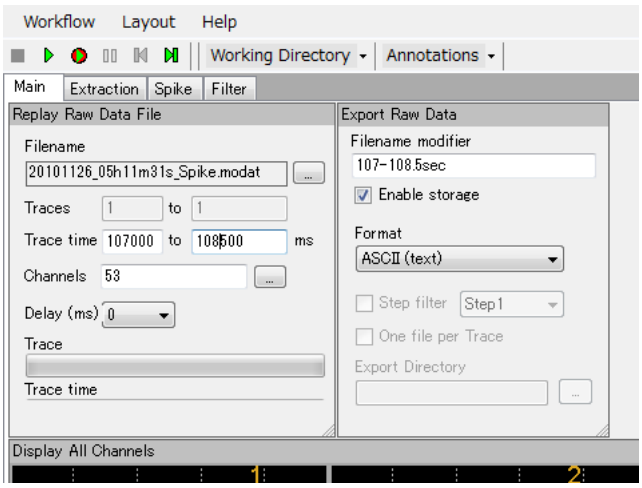
Mobius can export data in user friendly formats for analysis with other software.

Data to be exported	Module	File type
Raw data	Export Raw Data	Binary / ASCII
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].



File Format Version	20071201
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107000.05	-0.006836146
107000.1	-0.001953185
107000.15	-0.004394665
107000.2	-0.001953185
107000.25	0.000976592
107000.3	-0.005371258
107000.35	-0.008301035
107000.4	-0.002929777
107000.45	-0.002929777
107000.5	-0.004394665
107000.55	-0.007324442
107000.6	-0.000976592
107000.65	-0.001464888
107000.7	-0.007324442
107000.75	-0.003906369
107000.8	-0.002441481

Figure 10. [Left] Setting for exporting raw data with the Mobius (left). 107000-108500 ms of ch 53 are selected in the [Replay Raw Data File] module while ASCII is selected and Enable storage is checked in the [Export Raw Data] module. Data will be exported by clicking the green/red button.
[**Top-right]** A part of raw data exported by ASCII and displayed in Excel.
[**Bottom-right]** Waveform created by using Excel.

4-6. Burst analysis using the Mobius Offline Toolkit (MOT)

Burst analysis can be performed using the Mobius Offline Toolkit (MOT) software. The MOT read **Time Samp data saved by Mobius** and perform 2 types of burst analysis.

Analysis for Synchronized burst (array-wide bust)

The MOT's [Muli-channel Burst Analysis] program analyze so-called Synchronized burst (array-wide burst). It detect array-wide spikes as "synchronized bursts" and perform following analysis:

- Display and export Raster plots.
- Provide informations for the synchronized burst such as:
    - Number of burst.
    - Burst duration.
    - Burst rate.
    - Start time for a burst.
    - Inter burst intervals.
    - Number of spikes in a burst.

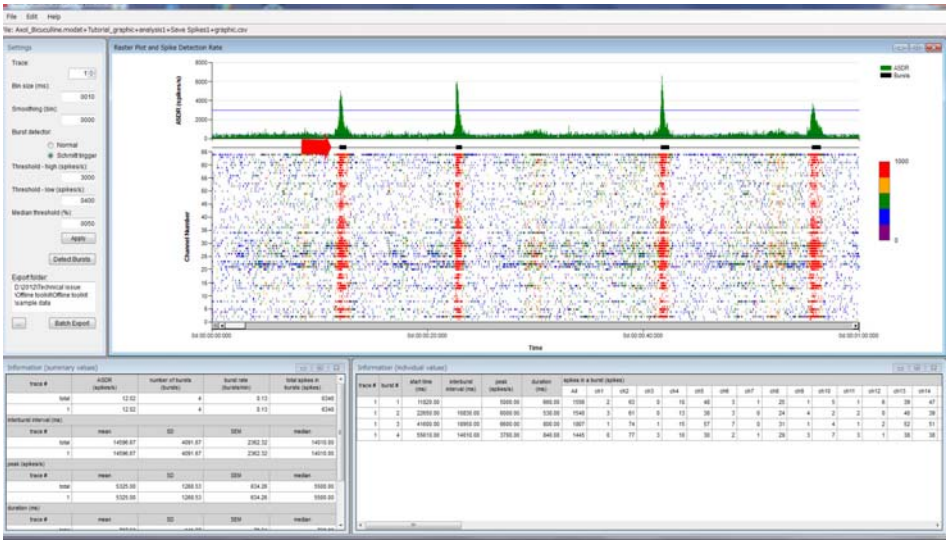

Figure 11. Synchronized burst analysis performed by MOT. Raster plot for spike timestamps are displayed on the top-right. Synchronized bursts are displayed in the black bars (red arrow in the middle of the Raster plot chart) and their information is provided in the charts (bottom).

Analysis for Single channel burst

The MOT's [Single Channel Burst Analysis] program analyze bursts for each independent channel. It detects high-frequency spikes in a channel as a Burst and perform following analysis:

- Displays Time course of spike events (time stamp).
- Computes and displays information for Bursts such as:
    - Number of bursts.
    - Burst duration.
    - Burst intervals.
    - Onset and offset time for a bursts.
    - Number of spikes per burst.

Figure 12. Single burst analysis performed by MOT. Time course of spike time stamps are displayed (top). Red shows "busts" extracted. The information for the "bursts" are displayed graphically (middle) and as a table (bottom).

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Alpha MED Scientific Inc.
Saito Bio-Incubator 209, 7-7-15, Saito-asagi,
Ibaraki, Osaka 567-0085, Japan
Phone: +81-72-648-7973 FAX:+81-72-648-7974
http://www.med64.com support@med64.com

Manufactured by **Alpha MED Scientific Inc.**

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