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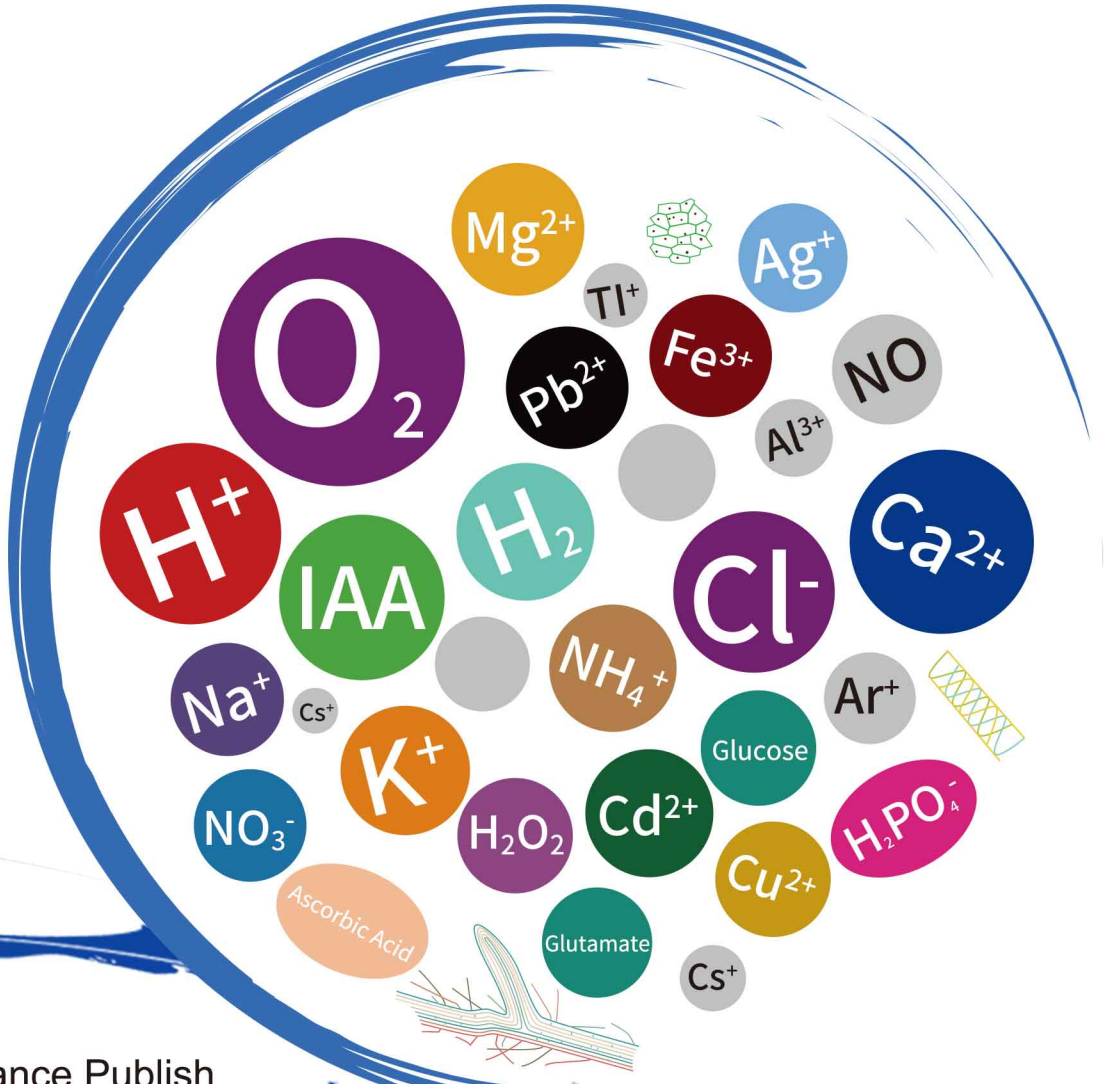

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2.1.2 Fluxes & voltage differences

The voltage differences stemming from the two-point measurement scheme of NMT is a key to understand how NMT works and why it can overcome the drifts of micro-sensor voltage readings **(Figure 2.)**. For instance, the voltage readings V_1 from 'Position-1' (solid line) will keep drifting even the measured ion's concentration is not changing at all. This drift makes it impossible to detect small concentration changes which forms the ionic and/or molecular concentration gradient resulted from their cross-membrane transportations.

However, by using the same NMT micro-sensor with the same drifting pattern in 'Position-2', dashed lines in Figure Voltage Differences Cancel out NMT Micro-sensor Electrical Drifts, $_{\Delta}V$ (V_1-V_2) will cancel out these drifts. Namely, if $_{\Delta}V$ is not '0', then there is an ionic concentration gradient, and the magnitude of flux is directly proportional to the $|_{\Delta}V|$. Moreover, the '+' and '-' signs of $_{\Delta}V$ determines the motion directions, efflux or influx of the ions/molecules we are measuring respectively.

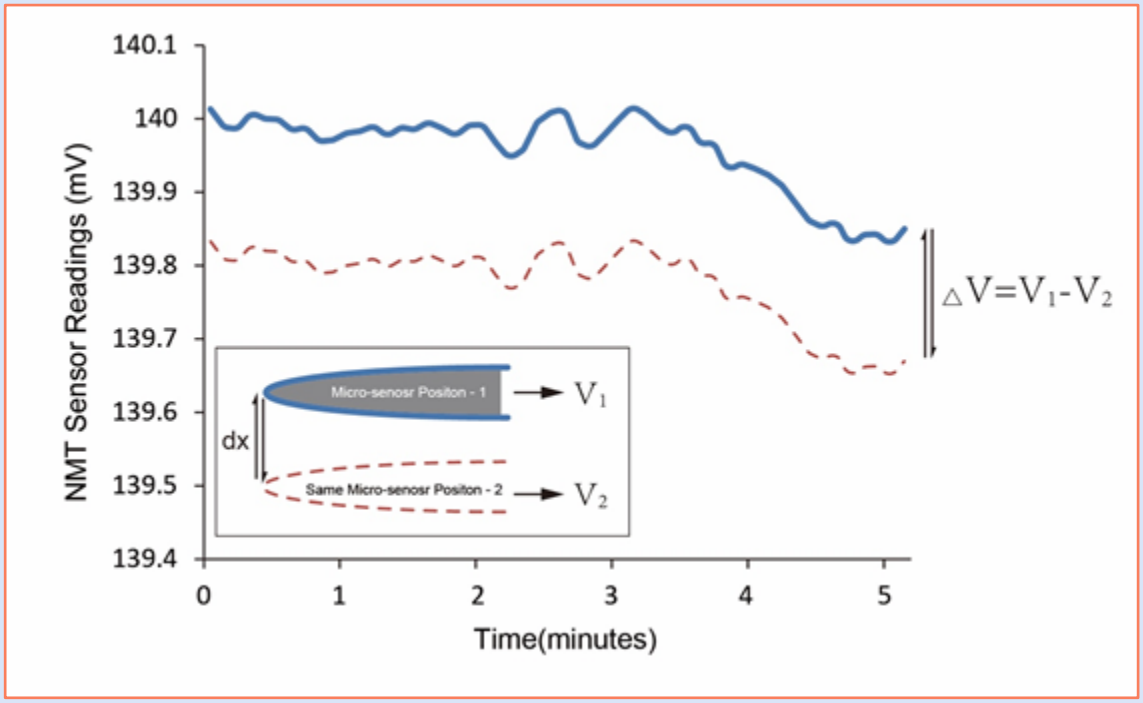

Figure 2. Voltage Differences Cancel out NMT Micro-sensor Electrical Drifts. Relationship between $_{\Delta}V$ and drift readings of NMT sensors. A real time course plot of a NMT sensor millivolts reading from 'Position-1 (solid line)' to 'Position-2 (dashed line)'. V_1 from 'Position-1' and V_2 from 'Positon-2' will be read constantly in a 'back-forth' vibrating pattern of movement around 0.3Hz frequency. $_{\Delta}V$ is calculated at the end of each cycle of sensor movement. **Insert:** shows the same sensor moving from Position-1 to Position-2 generating V_1 and V_2 respectively at distance dx.

2.1.3 Artificial ionic/molecular source tests & dynamic calibrations

The dynamic nature of NMT flux data requires a special way of calibration to make sure the NMT system is functioning as we expected, and the signals are real ones from the live samples we are measuring **(Figure 3.)**.

An artificial source, made with glass micropipettes filling with usually 100x higher ionic/molecular concentration comparing to its surrounding solution, is used to generate a sphere ionic/molecular gradient **(Figure 3a.)**. Then all parts of a NMT system, hardware, software, NMT micro-sensors, sampling rule(s), testing media composition et al., will be tested/calibrated while the sensor moves away from the tip of the artificial source.
A hyperbola fitting curve is expected with the data collected when the sensor is moving away from the artificial source **(Figure 3a.)**, because the theoretical ionic/molecular concentration gradient shall be consistent with a hyperbola curve.

Only replacing the artificial signal source with the real live sample to be measured when a real experiment is underway, so it is assured that the signals collected from the NMT system are coming from the live sample only **(Figure 3b.).**

2.1.4 Physical & chemical spaces

To better understand how NMT works, it is helpful to dissect the NMT operation space into physical space and chemical space respectively **(Figure 4.)**. The absolute coordinates (x,y,z) of every measuring point in physical space is very important to correlate ionic/molecular flux data back to the specific locations where the ions/molecules activities happened surrounding the live sample.

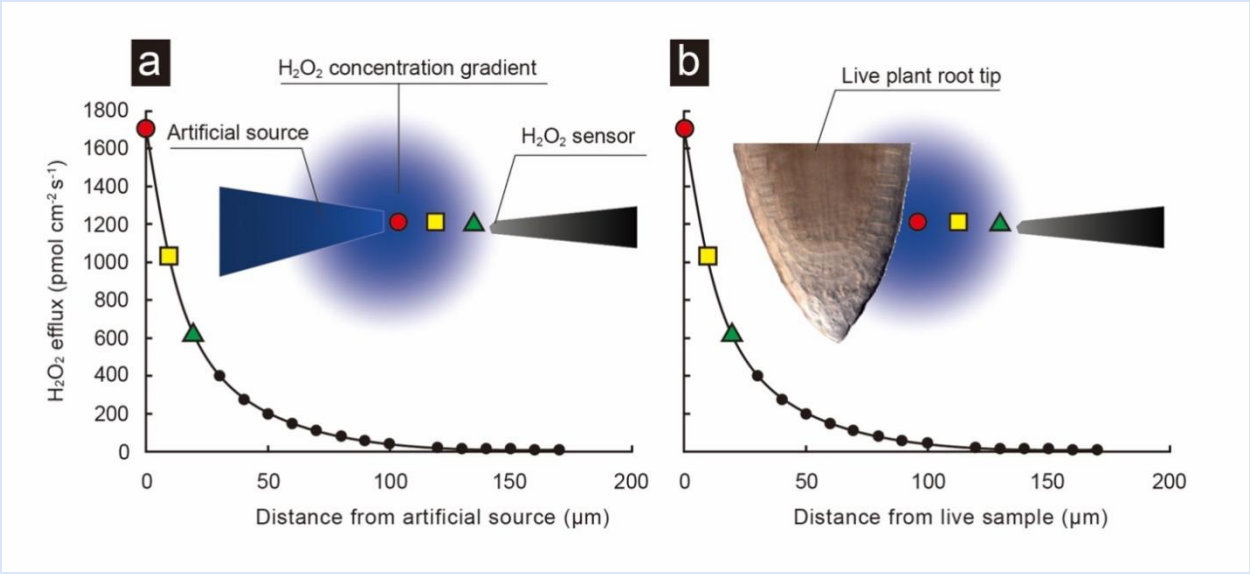

Figure 3. NMT Artificial Source Test & Dynamic Calibration. NMT Artificial Source Tests & Dynamic Calibrations. Using H_2O_2 sensor and a root tip as examples to illustrate how artificial source tests and dynamic calibrations work. **a:** a sphere H_2O_2 concentration gradient is formed near the tip of a glass micropipette filled with 100mM H_2O_2. A fitting curve is plotted according to the data collected while the sensor moves away from the tip of the artificial source (only three points (▲●■) are drawn); **b:** The artificial source is replaced with a live root tip sample, the same three points (▲●■) are measured.

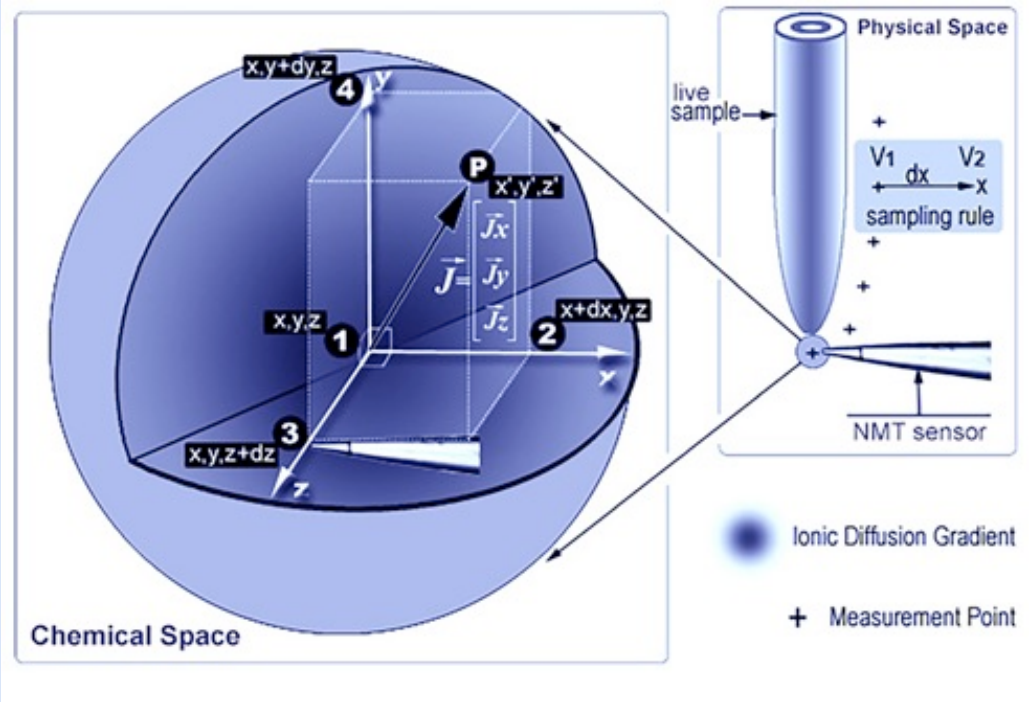

Figure 4. Physical and Chemical Spaces of NMT Working Space. Physical and chemical spaces in NMT working system. Two coordinate systems are artificially defined to make the relationships among the samples, measuring points of the NMT sensors, sampling rules and 1D, 2D, 3D fluxes detection easier to understand. **Physical Space:** measuring points '+' are located near the surface (usually between 2~10μm) of a root live sample, in which a NMT sensor makes two-point flux measurements according to a predefined 'sampling rule'. A sampling rule determines the dx (usually between 5~50μm), the distance between V_1 and V_2 (V_1 and V_2 are explained in **Figure 1.**) readings collected, the time needed for the NMT sensor to travel the dx, and waiting times the sensor has to keep motionless in the two-points in x-axis direction, or in multiple axis directions illustrated in chemical space.. **Chemical Space:** It is an enlarged symbolized illustration of a measurement point '+' in physical space. It uses color gradient to represent ionic/molecular diffusion gradients when they go in or come out of a live sample. Point 1, with different values in its physical space coordinates system, is the origin of its chemical space Cartesian coordinate system. NMT allows sensor(s) to move in x, y and z directions so that the ionic/molecular fluxes in real scenario of living environment, from point 1 to point P, are able to be measured and calculated, namely values with both magnitude and direction information.

Chemical space refers to completely different coordinate systems in which the zero points are overlapping with the zero points of each measuring point from the physical space **(Figure 4.)**.
The values of xyz in chemical space can be translated into the physical space coordinate system which is very useful for flux data 3D visualization.

2.1.5 1D, 2D, 3D and 6D measurements

One of NMT's liberties due to its no-touching way of operation is that NMT can maneuver freely in its physical space **(Figure 4.)**, which gives huge leverages to help scientists to collect ionic/molecular flux data in multi-dimensions. Ionic/molecular fluxes per se are values with directional information embedded, by which is the true nature of any live biological samples who should give rise to 4-dimensional flux information at any time being measured.

Meanwhile, flux data in 1D, 2D and 3D are essential tools to a) validate results from molecular biology, biochemistry, microscopy

and omics experiments; b) provide cues for future research looking for new genes, new mechanisms; c) connect the dots to form a network map reflecting the inter-relationships among genes, proteins, metabolites et al.

An online interactive 3D animation application has been developed to help researchers to not only visualize 3D flux data but also play with some features, such as zooming, spinning, panning, movie making and coloring etc. (**Figure 5.**)[27]

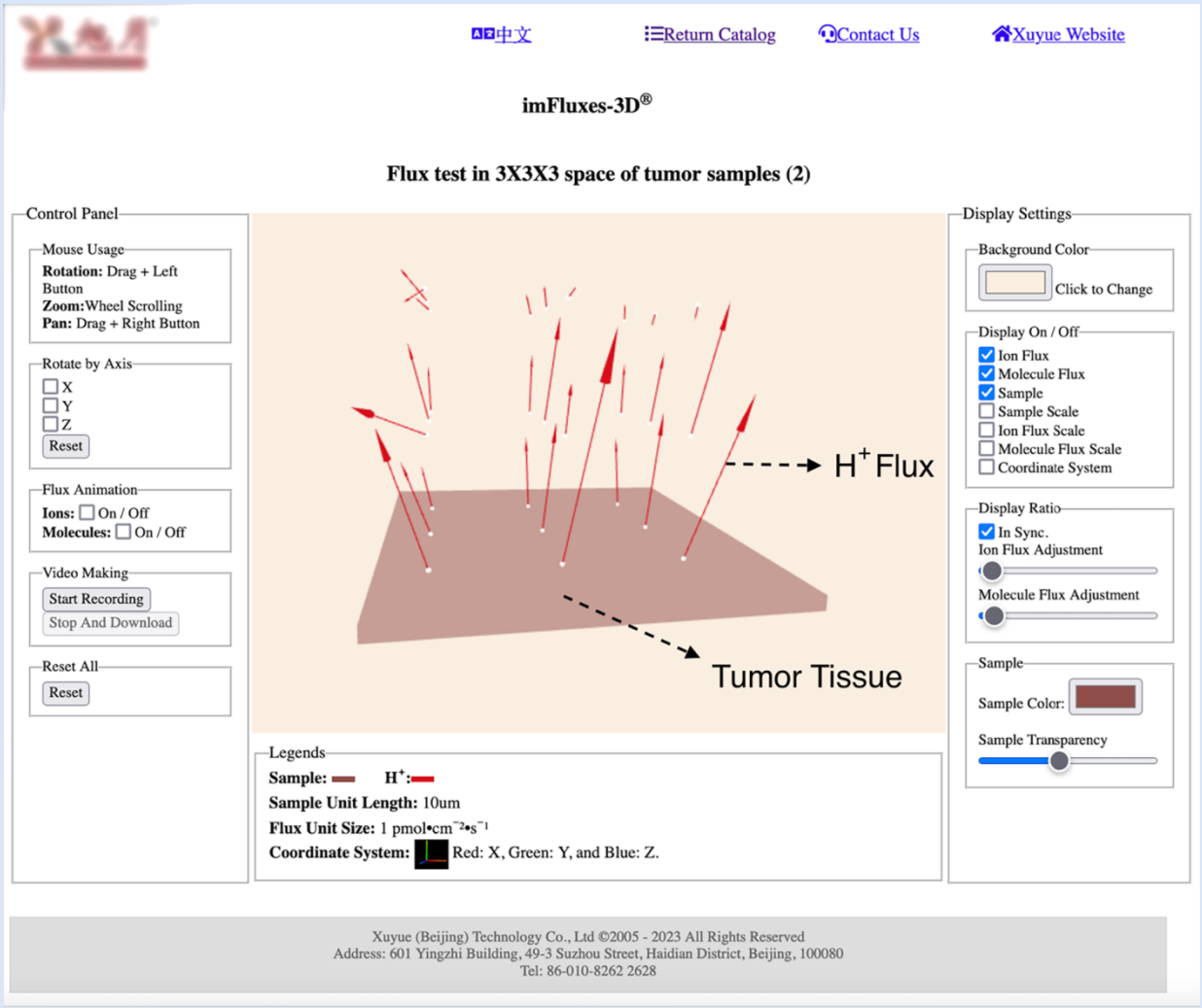

Figure 5. 3Danimation. Screenshot of imFluxes3D® online version. **Top**: navigation area and title of current 3D file displayed; **Left:** 'Control Panel': 1) 'Mouse Usage' explains how to use mouse for this application; 2) 'Rotate by Axis' allows users to spin the 3D animation along x,y,z axis individually or in any combinations; 3) 'Flux Animation' shows the flux vector in a dynamic fashion; 4) 'Video Making' enable user to record a piece of animation and save it into user's local computer in mp4 format; 5) 'Reset All' resets the whole application to initial state; **Middle:** Area for displaying the 3D animation of samples, ionic/molecular fluxes and legends. It will be updated in real time as user adjusts the parameters in both 'Control Panel' and 'Display Settings'. **Bottom:** legends to the 3D animation above is displayed here; **Right:** 'Display Settings': 1) 'Background Color' clicks to change the background color of the 3D animation; 2) 'Display On/Off' controls the display of components of 3D animation; 3) 'Display Ratio' arbitrarily adjusts the size of the ionic and/or molecular flux vectors either in sync or independently; 4) 'Sample' determines the color and transparency of the sample displayed in Middle.

A recent development of NMT hardware design allows both NMT micro-sensor and the tested sample to move in their own 3D system independently, which presents scientists a even more powerful and versatile 6D NMT system [9].

2.1.6 High-throughput NMT in AI Era

It had long been a dream of NMT community to push it into the high-throughput category of technique since it was born. The challenges are: a) the efficiency and uniformity of NMT sensors to construct and to perform; b) the physical limitations imposed by the size of NMT sensors restricts the number of ions/molecules that can be detected simultaneously; c) accurate positionings of NMT sensors relative to the live samples being measured in 4D; d) live sample preparations and customized NMT system setups according to the unique characteristics of each live samples.

The booming of Artificial Intelligence (AI) has been making big impacts to the R&D and production of NMT, which also paves the way for the birth of high-throughput NMT **(Figure 6.)**.
For example, like the human face recognition function of various gate-control people are using daily, the widely used image recognition technique of AI has been adopted into NMT, which overcome the difficulties to put both NMT sensors and live samples at required precise positions in a 4D (xyz + time) coordinate system under microscopes **(Figure 6.).**

As more AI functions integrated into the R&D process of NMT, more customized high-throughput NMT system will be available for specific research areas in the foreseeable future [9,28,29].

Figure 6. AI's Applications in NMT. Any combinations of any number of NMT ion(s)/molecule(s) microsensors are doable now due to the adoption of AI sampling rules. **a:** A High-throughput aiNMT System can measure seed vigor in a 12-well plate in a fully automatic fashion; **b:** A dual-sensor configuration was used to simultaneously measure both H+/O2 fluxes of a tumor tissue; **c:** Illustration of AI-based automatic sample image recognition and testing point locating; **d:** AI-based NMT microsensor making instrument by which a tiny volume (ca. one millionth of a drop of water) of liquid ionophore can be manipulated accurately.

2.1.7 Gradraw® & NMT visualization

Data visualization is a tendency to make research more efficient in scientific instrument sector. Gradraw® is a new NMT technique of visualizing ionic/molecular concentration gradients aiming to make researchers to comprehend changes of ions/molecules easier [30].

As mentioned earlier, fluxes and gradients of ions/molecules are very close inter-related. Being results from fluxes, ionic/molecular gradients have been found not only easy to understand, but also more straight forward to put into the context when scientists try to explain the causes and/or results from ionic/molecular activities involved with other types of data from other techniques (**Figure 7.**).

2.2 Limitations of NMT

There are a few limiting factors such as NMT micro-sensor varieties, temporal and spatial resolutions in addition to the fact that NMT cannot, in most cases, penetrate sample barriers to obtain reliable readings of ions/molecules inside samples.

2.2.1 NMT Micro-sensors

Knowing exactly which ions/molecules you measuring is very straightforward of NMT users, but adding new members to the current NMT sensor's list **(Table 1.)** is not an easy task, unfortunately. For examples, some breakthroughs have been made to the most wanted $Fe^{2+/3+}$ and PO_4^{3-} sensors recently for years of struggles and efforts. The reasons that this progress has been slow is a) only a handful of scientists around the world are working in

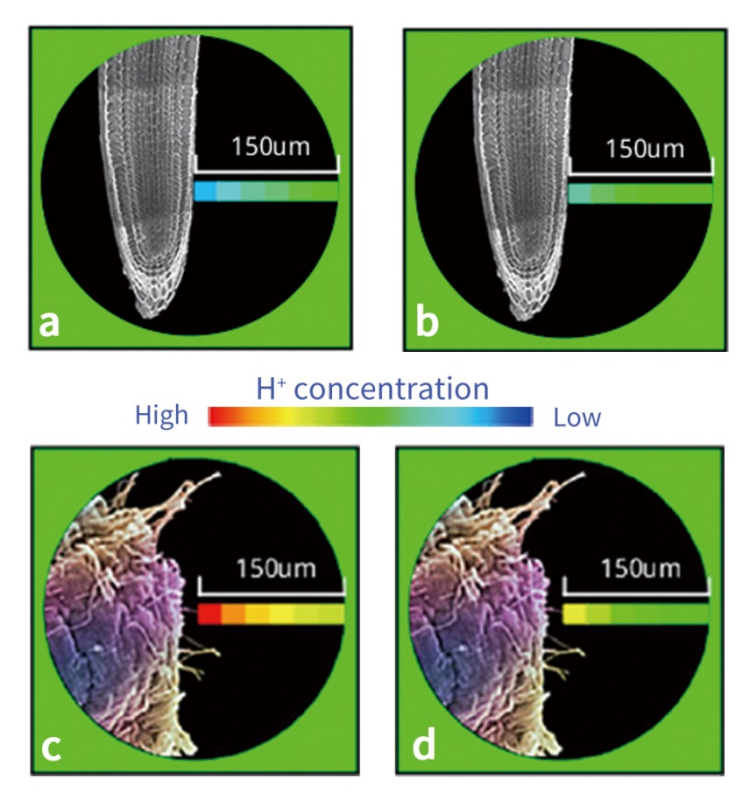

Figure 7. Example Results from Gradraw® Technique. Representative results of Gradraw® technique. **a)** 30 seconds after cold treatment with a *Arabidopsis* root; **b)** 120 seconds after cold treatment with the same sample; **c)** fresh breast cancer tumor tissue; **d)** 10 minutes after a chemo-drug applied to the same sample.

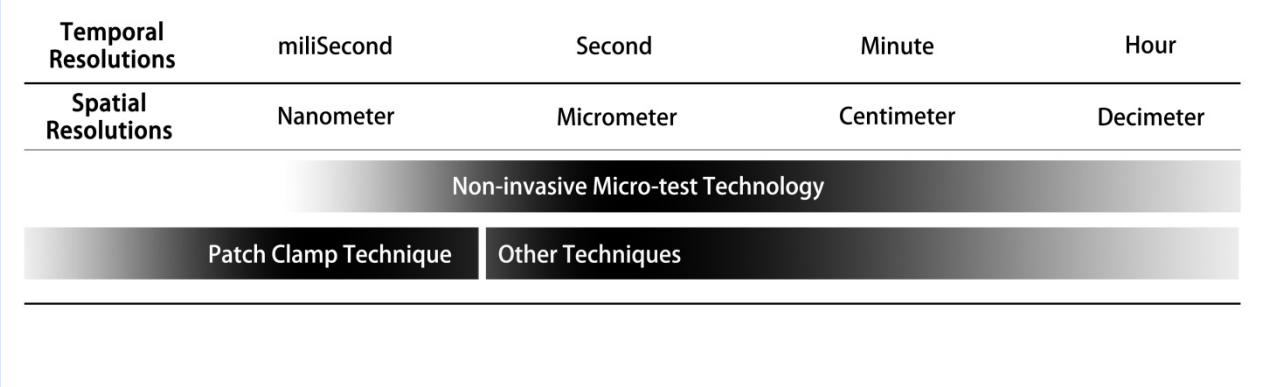

Figure 8. NMT Temporal and Spatial Resolutions. Temporal and spatial resolution comparisons between NMT and other techniques. NMT resides the range covering the gap between patch clamp and other techniques.

this specialized field; b) due to the inter-disciplinary nature of NMT sensor's development, close collaborations are crucial to the final success; c) lacking driving force from commercialization results in unwillingness to develop new NMT sensors for both academic and business sectors.

2.2.2 Temporal resolution

NMT's temporal resolution is mainly determined by its responding time, which is also one of the reasons why NMT micro-sensor is difficult to develop. Because while obtaining reliable and stable readings, the NMT sensor requires responding time in a few seconds range versus dozens of seconds or even minutes of conventional micro sensors.

Although temporal resolution may be a limiting factor for NMT to go below seconds, biologists are taking advantage of its relative long term detection capability to up to hours or even days, which is also very much desirable, because life lives in multiple temporal domains. **(Figure 8.)**.

2.2.3 Spatial Resolution

It's common for a researcher who wants to detect ionic/molecular fluxes from live samples as small as possible. Spatial resolution in micro-meter (μm) scale of NMT is not very impressive comparing to many other nano techniques. Yet, due to the ability to make measurements without touch the samples, collective flux signals can be detected from centrifuged/condensed small samples like chloroplasts/mitochondria/bacteria et al.

2.3 Comparative advantages with other techniques

Every technology is unique in a way that it was invented to meet both challenges of science advancements and compromises to what top technologies were capable of at specific times of natural science development. Although almost all technical paths may lead a scientist to the Rome s/he expects to be, picking up the right one is crucial to make sure to get there earlier than others. Therefore, understanding the differences among different technologies that better fit someone's research is important.

2.3.1 NMT vs. Micro-electrodes

One common misconception about NMT versus traditional Ion Selective and/or molecular specific Micro-electrodes is that people think these are the same thing. On one hand, it is true to certain extent because they both tell you the information about the ions

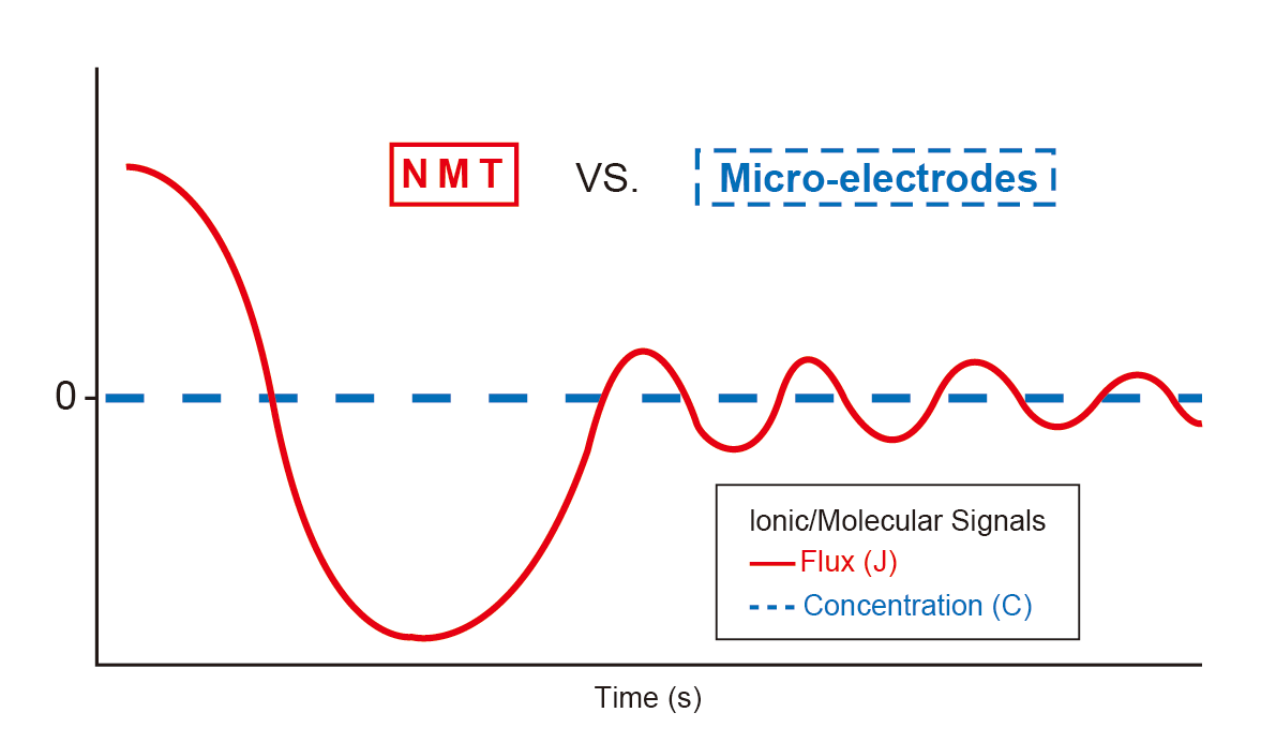

Figure 9. NMT versus Micro-Electrodes. Comparison between NMT and Micro-electrodes. Under same experimental conditions, NMT can pick up reliable and quantifiable ionic/molecular flux signals while micro-electrodes cannot.

and/or molecules you are measuring, on the other hand, the big difference relays upon how much information each technique can provide **(Figure 9.)**. Obviously, NMT can give you more insights about the biological phenomena you would like to explore.

As matter of fact, they are not even comparable to each other because NMT ionic/molecular fluxes give you dynamic values with embedded directional information, whereas micro-electrode can only give people static concentration with much lower sensitivities comparing to NMT.

2.3.2 NMT vs. Patch Clamp

Nobel Prize of Physiology/Medicine was awarded to E. Neher and B. Sakmann in 1991, only 15 years after they invented patch clamp (PC) technique which reveals ion-channel functions by measuring ionic currents in the plasma membrane of living cells [31].

One of the major breakthroughs with PC is the successful GigaΩ seal between the polished glass micro-pipettes and the plasma membranes by which allows the ionic currents to be detected. Yet, it also brings in significant mechanical impacts or even damages to the live cells studied, which in turn makes the mechanical stimulations to be an inherent factor throughout every PC experiments [32].

Therefore, NMT, an non-invasive way of detecting ions across cell membranes is very attractive to biologists. Especially for plant biologists who wants to study ions, because they don't have to enzymatically peel away the cell walls of plant cells which undoubtably changes the properties of cell membranes significantly [9].

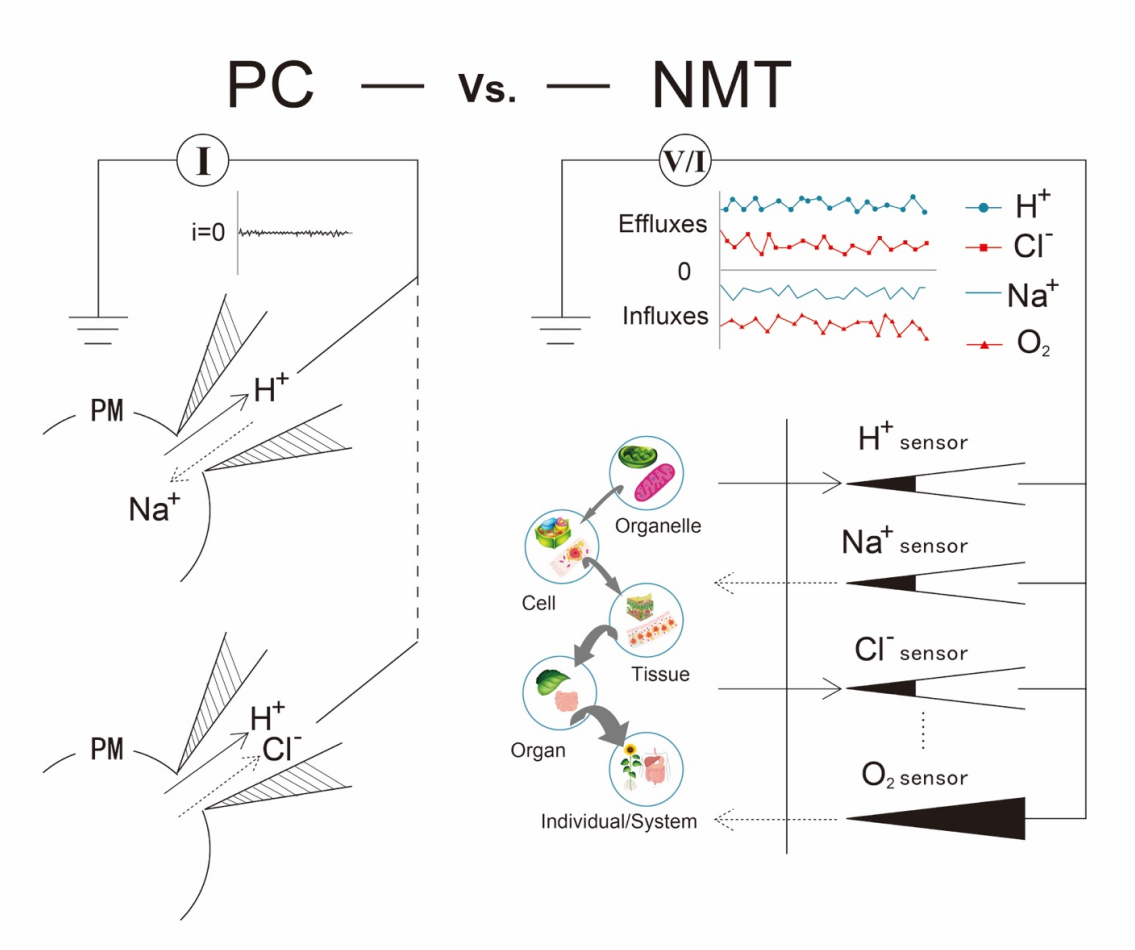

Figure 10. NMT versus Patch Clamp. Comparison between NMT and Patch Clamp technique. **Left Panel:** There are no currents when two same electrical charged ions moving in the opposite direction, as well as two opposite electrical charged ions moving in the same direction. **Right Panel:** Regardless of the sizes of the samples and their electrical charges, as long as there are specific ionic/molecular sensors available, NMT can pick up all the ionic and molecular signals from them. H^+, Na^+, Cl^- and O_2 sensors are used as examples to illustrate not only the different types of NMT sensors can be used simultaneously with different samples, but also effluxes and/or influxes of multiple ions/molecules can be detected at the same time in one NMT experiment.

Be able to know what ion(s) is being measured even before a researcher starts a NMT test is quite convenient versus PC. Plus NMT can also detect ion(s) movement(s) even under the electrical neutral conditions in which PC will not be able to measure (**Figure 10.**)

A valuable by-product of no-touching measurements of NMT is that it can measure a wide range of live samples in different sizes, from small intact organisms, isolated organs, tissues, cell layers, single cells to condensed organelles or microbes.

Detection of multi-ions/molecules simultaneously is another unique feature of NMT, which allows scientists to study the inter-relationships not only among ions but also among ions and molecules potentially paving the way to decipher physiological phenomena in various forms of life [33,34].

However, PC still have the leverage versus NMT with respect to both temporal and spatial resolutions **(Figure 8.)**, which allow scientists to take advantage of both techniques to tackle tough scientific questions [35–37].

2.3.3 NMT vs. Fluorescence Microscopy

Fluorescence Microscopy is a powerful technique enable scientists to detect both the presence and localization of fluorescent ions/molecules in live samples. So, theoretically people can detect any ions/molecules as long as they can be labeled by a fluorescent molecule which can be detected by a fluorescence microscope.

However, from NMT's perspective, there are a few technical pitfalls of Fluorescence Microscopy users need to pay attentions to: a) invasive labelling processes: the micro-injection and dye diffusion are imposing physical damages and chemical disturbances respectively to the samples; b) photo bleaching: not only complicates the fluorescent dyes' signals detected but also prevent it from long time observations; c) buffering effects: ions/molecules are deprived by dye labelling from the physiological process of the live sample being studied, therefore the ions/molecules are buffered which may give rise to unpredicted results **(Table 2.)**.

Therefore, it has been a common practice nowadays for scientists to use Fluorescence Microscopy to measure ions/molecules inside samples while using NMT to detect ions/molecules outside [38,39].

2.3.4 NMT vs. Radioactive Labeling, NMR, Gas Chromatography, Cyclic Voltammetry and Nano SIMS

Techniques aiming to detect ions and/or molecules have been developing rapidly in recent years. Here we are making comparisons between them and NMT with intention to providing researchers a reference to pick up the right technique(s), NOT the best one(s), for their unique research **(Table 2.).**


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