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Mechanism and Applications of Plasma Gene/Molecular Transfection

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instead of Conclusion

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1st Type : Energy Conversion/Generation

Plasma specialists

Generate something

Light Sources

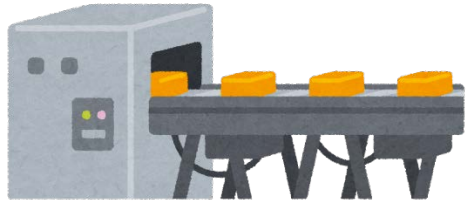
Fusion

2nd Type : Material Conversion

with Background of Physics/Chemistry

Produce something

Change the structure



Plasma Deposition

Plasma Etching

Plasma Torch

3rd Type : Process Trigger

Far from plasma unknown with fear

Indirectly induce something

Plasma activated X

Plasma assisted X

cf. Germination

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3. The Random Genome Integration-free
   - Spontaneous Uptake induced by complex of stimuli
4. The Applications and Future Tasks of the Plasma Gene/Molecular Introduction
5. Growth Acceleration of Fish using Plasma

What is Molecular / Gene Introduction? (or Gene Transfection)

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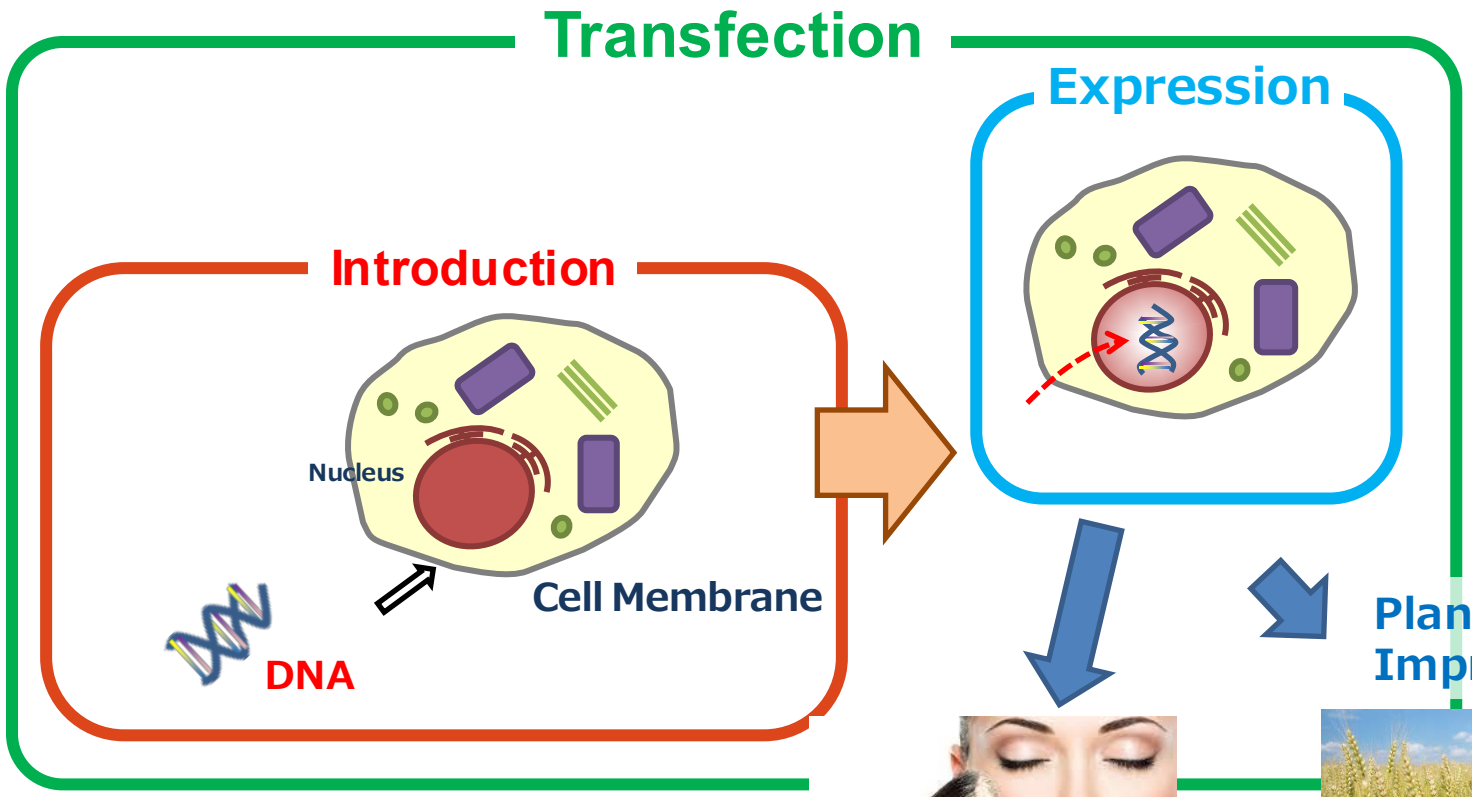
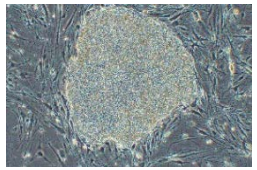
A technique to **introduce nucleic acids into cells** artificially for **expression of their function**.



Gene Therapy and Regenerative Medicine

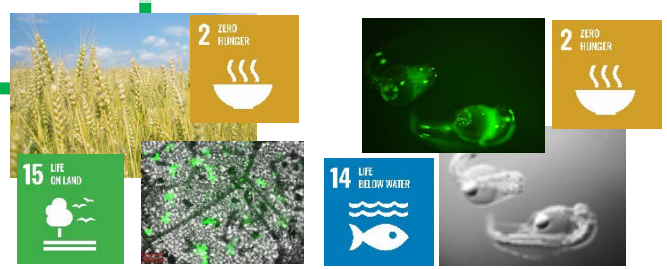


iPS

Drug Development

Genome Editing

Plant Breeding and Improving of Fish



Beauty and Cosmetics

Legacy Methods: How to Introduce / Transfect?

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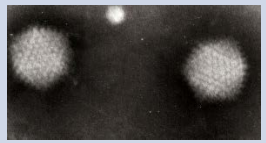
Chemical method	Physical method	Biological method
 Ex) Lipofection method	 Ex) Left: Gene gun method Right: Electroporation	bacteria  Adenoviruses  Ex) Left: Agrobacterium method Right: Viral vector method

Damage on Cells and Side Effects (Cytotoxicity, Immunogenicity, Antigenic)

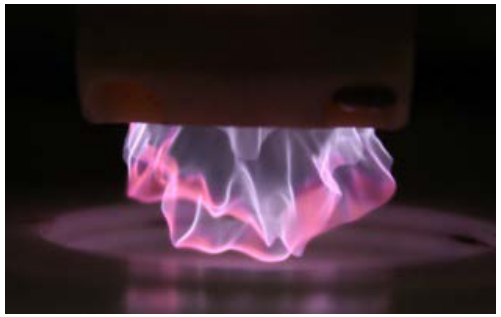
2002

Plasma Method

Invented by Fujisawa Pharmaceutical Co.,Ltd. (2002)

and Reported by Ogawa (2005)

*Y. Ogawa et al., *Biotechnology and Bioengineering*, **92** 865 (2005).



Proto-type

Advantages: High Transfection Rate and High Viability
Problems: Stability and Reproducibility

Seeking the Best Plasma

M. Jinno et al., *Japanese Journal of Applied Physics*, **55** 07LG09 (2016)

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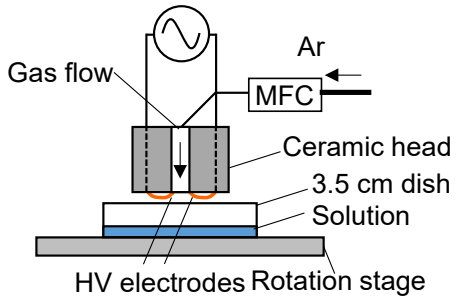
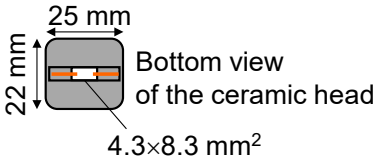
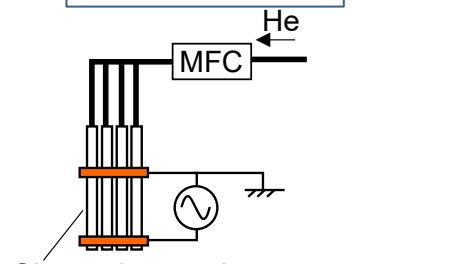
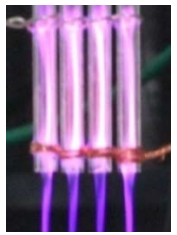
Arc (flare) plasma



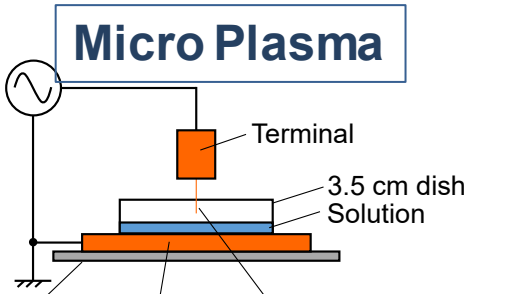


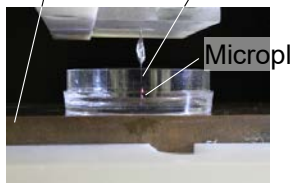
Plasma jet



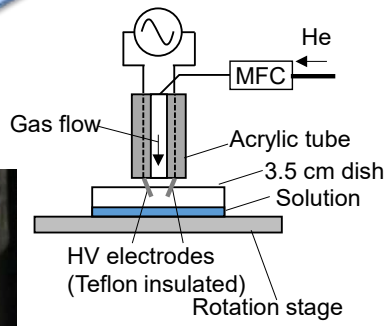


Micro Plasma

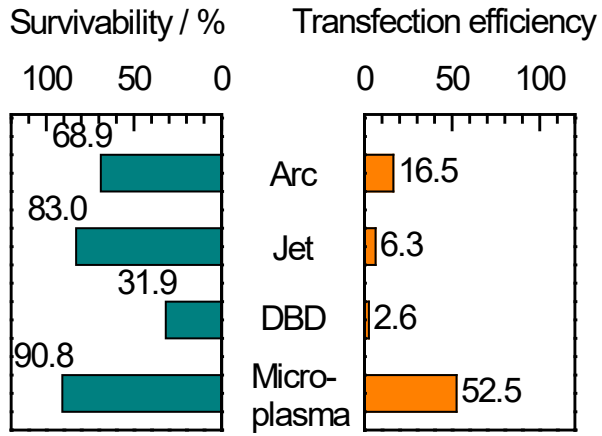




2002 → 2010 → 2012



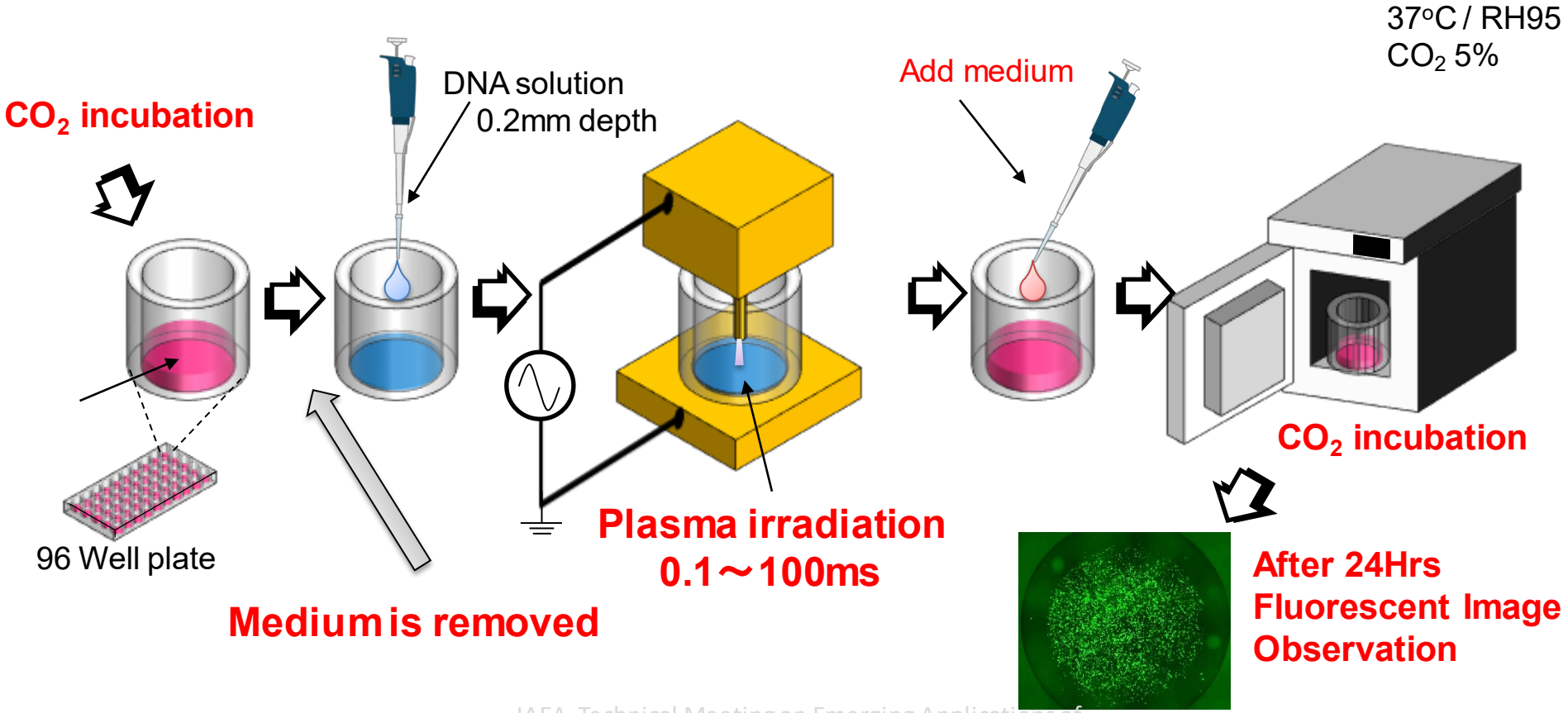
Dielectric Barrier Discharge



Protocol of Micro Plasma Method (Same for Surface-discharge method)

Experimental procedure

1. Put the 96 Well plate on the plasma gene transfection device.
2. Plasma irradiation.
3. Cell observation after cultured for 24 Hrs.



Plasma Gene Introduction

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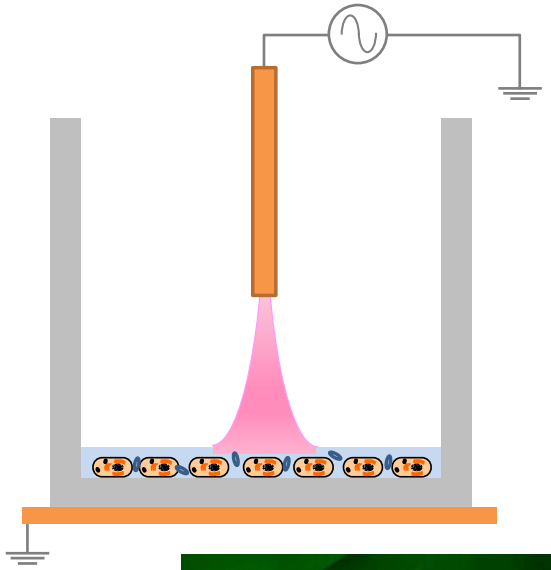

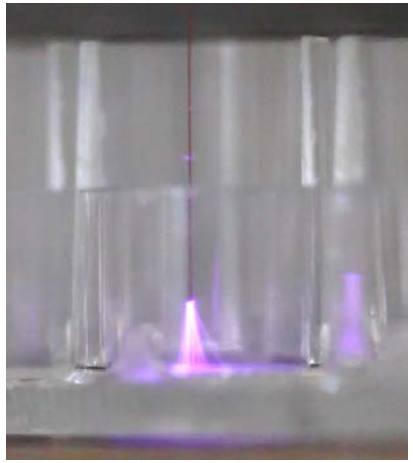

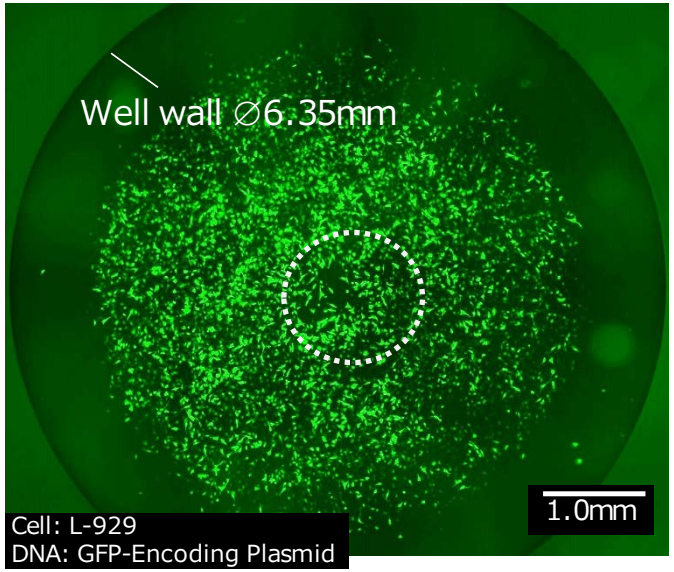

Unique Characteristics

- Cells are sandwiched by two electrodes.
- DBD type (Dielectric Barrier Discharge)
  Electric Current pass-through cells!
- Introductions occur in regions outside of the direct plasma exposure.
- Short treatment time 10^{-1}~100 ms
- High transfection efficiency over 80 %
- High viability of cells over 90%
- Not good for Mass-Processing

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The Mechanism : Complex of Electrical and Chemical Stimuli

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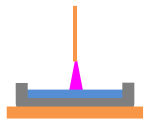
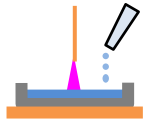
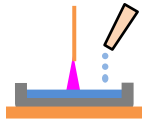
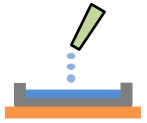
	MDP (micro-discharge plasma)	MDP with catalase	MDP with NAC	LPP (laser produced plasma)	H_2O_2
					
	Standard	Scavenge H_2O_2	Scavenge ROS	Remote Plasma w/o Electric Charge	Drop H_2O_2
Electrical Stimuli	✓	✓	✓	nil	nil
Chemical Factor	ROS (H_2O_2) other ROS other RS (RS: Reactive Species)	~~ROS (H_2O_2)~~ other ROS other RS	~~ROS (H_2O_2)~~ ~~other ROS~~ other RS	ROS (H_2O_2) other ROS other RS	ROS (H_2O_2) ~~other ROS~~ ~~other RS~~
Relative Introduction Efficiency	100	40	20	0	0

ROS is Required

Electrical Factor is Required with ROS

H_2O_2 is important in ROS

Complex of Electrical and Chemical (ROS) Stimuli are required!

How does the introduction occur?

The Mechanism: Complex of Electrical and Chemical Stimuli

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Directly controlled parameters

Voltage Current | Waveform | Electrode length | Working gas | Electrode construction | Irradiation time

Micro Plasma

Factors

Electrical
Electric field,
Current, Charge

Chemical
Reactive species
(ROS, RNS)

Effects

Electrical effects

Chemical effects

Processes on/in cells

Charge up Poration

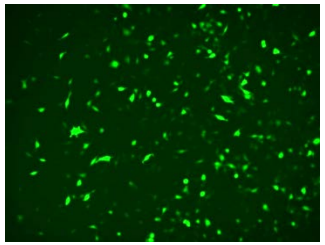
Cell activation
Biological Reactions

Peroxidation Poration

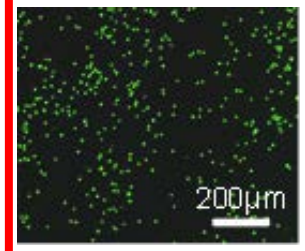
Endocytosis

Micro-Plasma

Plasmid DNA
3x10^6 Da

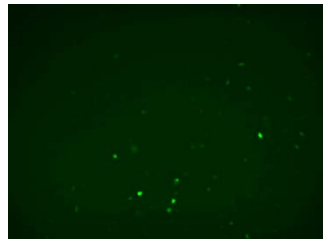


Middle Molecule
1270 Da



Endocytosis Inhibitor with Micro-Plasma

Plasmid DNA
3x10^6 Da



Middle Molecule
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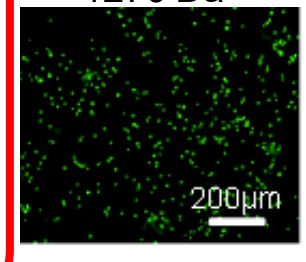

M. Jinno *et al. Arch Biochem Biophys.* **605**, 59-66. doi: 10.1016/j.abb.2016.04.013 (2016)

Z-Potential（Charge up of Cells）

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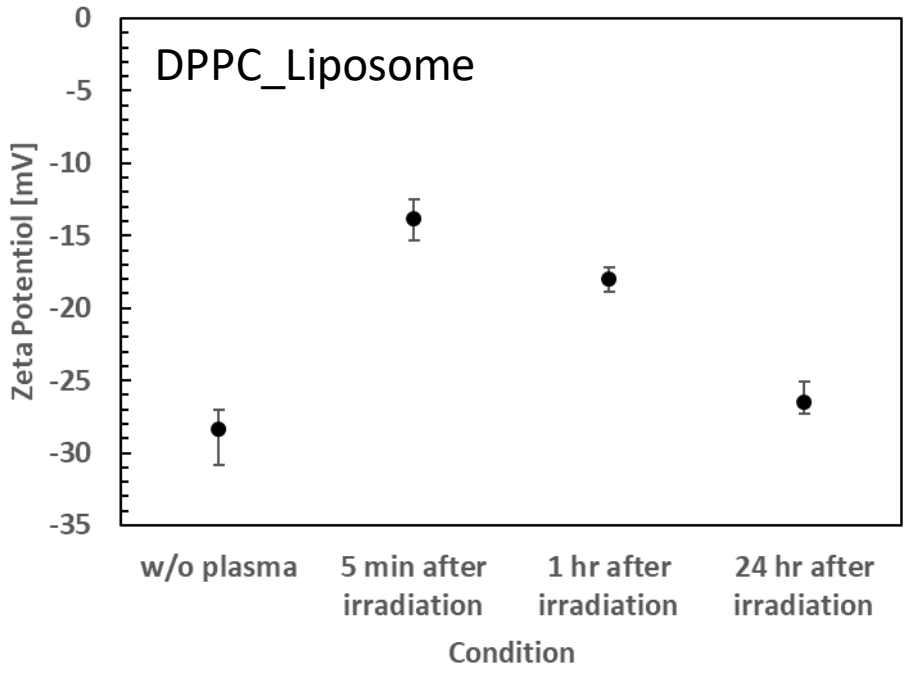

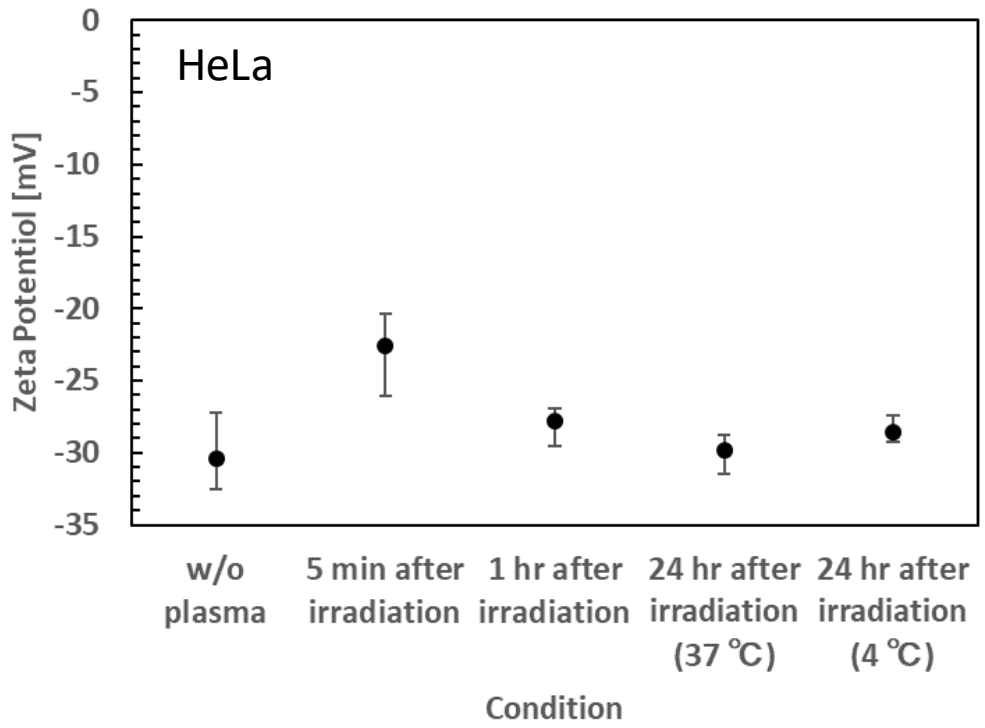

Target	Zeta Potential [mV]				
	w/o plasma	5 min after irradiation	1 hr after irradiation	24 irr	
				37 ℃	
HeLa	-30.4	-22.6	-27.8	-29.8	
DPPC_Lipo	-28.4	-13.8	-18.0	—	

Negative charge decreases after plasma treatment
→cell membrane locally charged up positively.
→ the collision between cells and plasmids increases and cytosis is enhanced.

The Mechanism: Complex of Electrical and Chemical Stimuli

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Effective Factors:

Metabolic effect of electrical and chemical factors produced by a discharge plasma※1

Endocytosis contributes more than 80% of introduction process※2

Effects of Chemical Factors

ROS triggers endocytosis※3

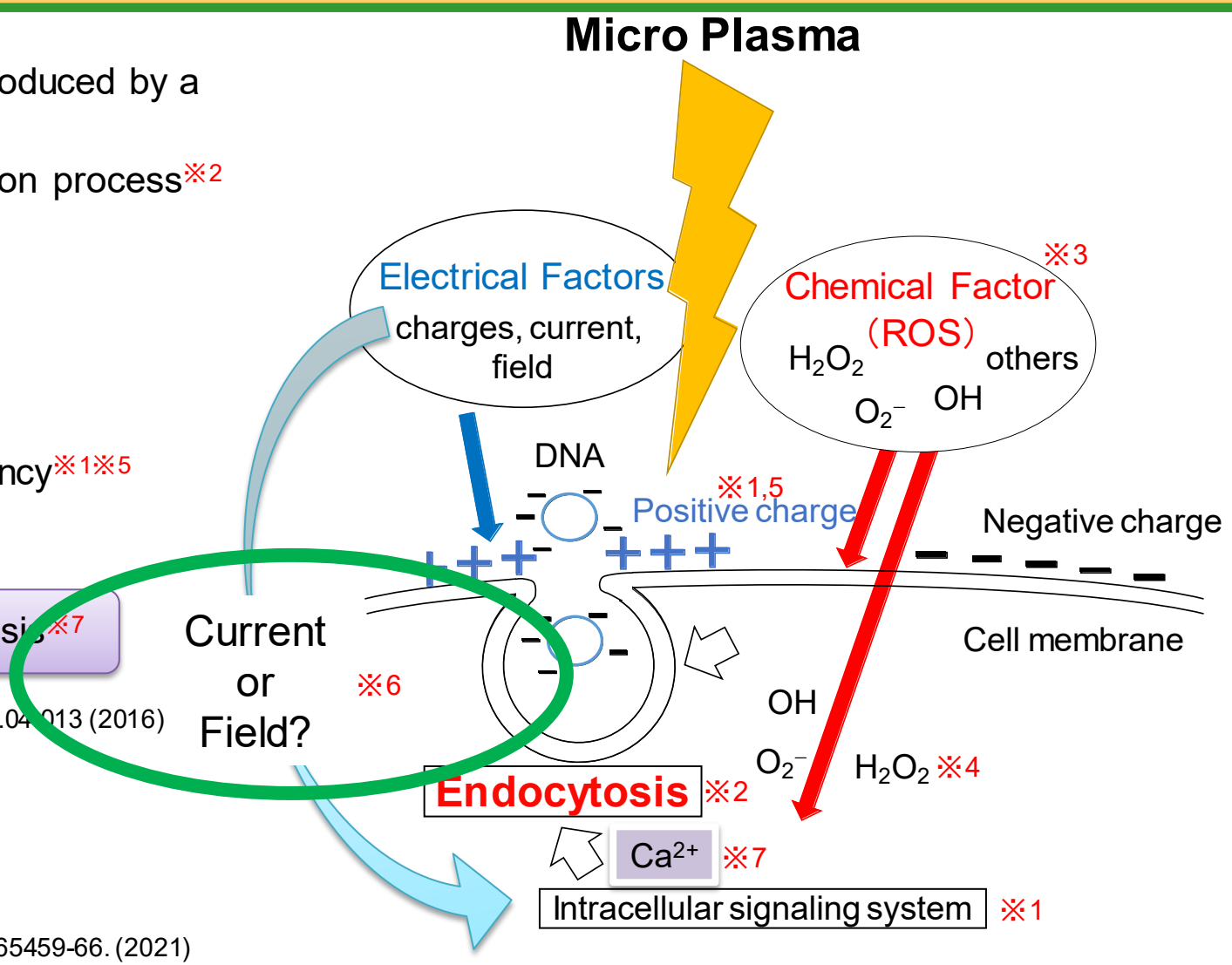
H_2O_2 is important in the trigger process※4

Effect of Electrical Factors

Charge up cell membrane increases collision frequency※1※5

Current is important for endocytosis trigger※6

Xin-Sheng Wu *et al* (2009) : Ca^{2+} Initiates Endocytosis※7



M. Jinno *et al. Plasma Sources Sci. and Technol.* **26**, 065016 (2017)

※1 M. Jinno *et al. Arch Biochem Biophys.* **605**, 59-66. doi: 10.1016/j.abb.2016.04.013 (2016)

※2 M. Jinno *et al. Plasma Sources Sci. and Technol.* **26**, 065016 (2017)

※3 Y. Isozaki *et al. Jpn. Plasma Medicine.* **7(4)**, 321-332 (2017)

※4 Y. Ikeda *et al. Jpn. J. Appl. Phys.* **55**, 07_LG06 (2016)

※5 T. Hiramatsu *et al. Jpn. J. Appl. Phys.* **58**, SEEG05 (2019)

※6 Y. Kido *et al. PLoS One.* **16(1)**, e0245654. doi: 10.1371/journal.pone.024565459-66. (2021)

※7 Xin-Sheng Wu *et al. Neurosci.* **12**(8): 1003–1010. doi:10.1038/nn.2355. (2009)

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Equivalent circuit network

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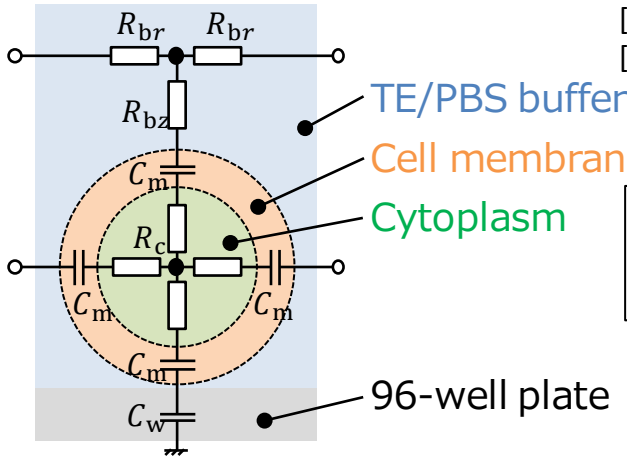
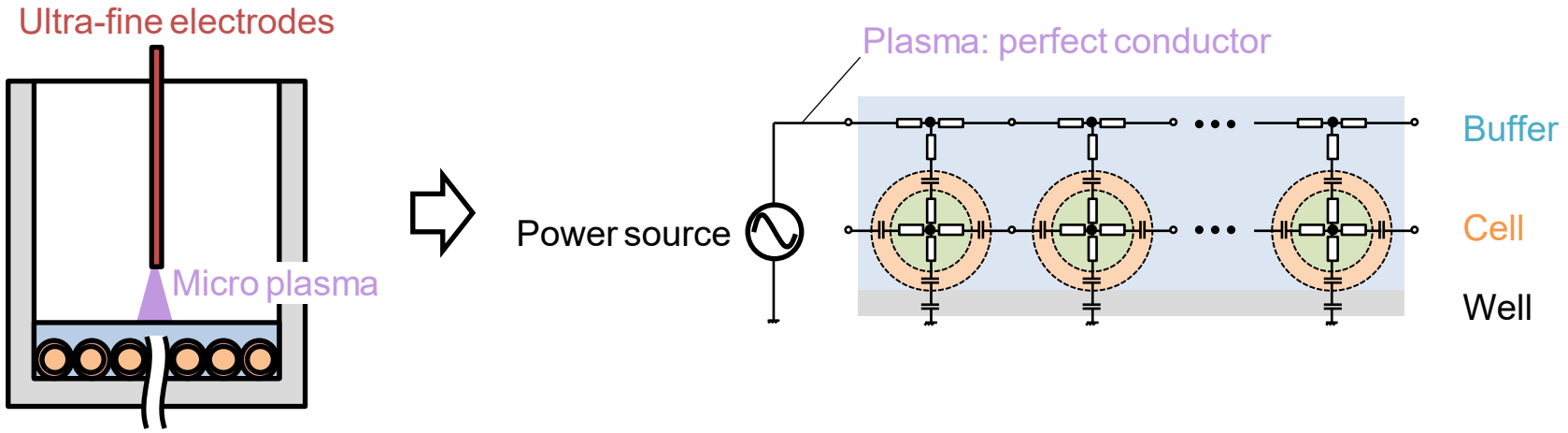
(1) Equivalent circuit expressing a single cell, buffer solution and bottom wall of well plate.



[5] I. Ermolina *et al*, IEEE Trans. Dielectr. Electr. Insulat. **8,** 253 (2001)
[6] N. Nomura *et al*, IEEE Trans. Dielectr. Electr. Insulat. **16,** 1288 (2009)

Cell membrane: Capacitor
Cytoplasm: Resistor

(2) Equivalent circuit network modeling buffer solution, cells, and a 96-well plate.



Equivalent circuit network

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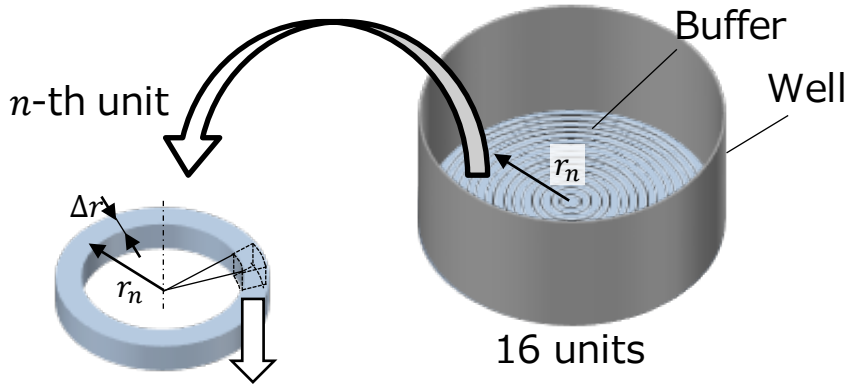
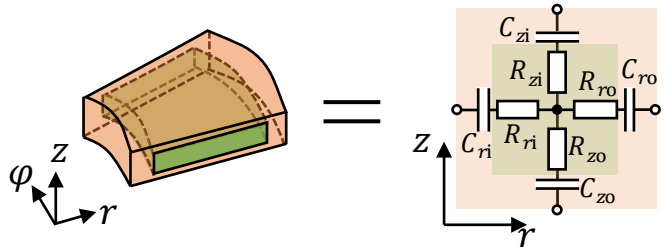
(3) Assuming axisymmetry



One element contains several cells.

⇩ Unite the cells



⇩

Radial 16 longitudinal connection

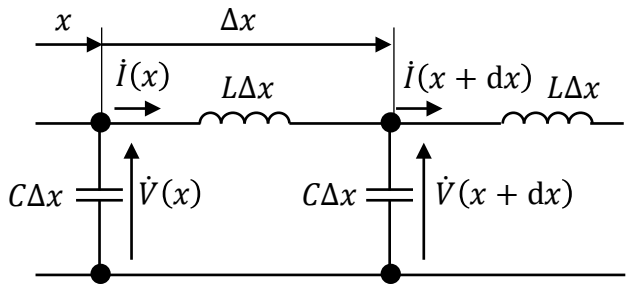
No need to consider circumferential direction

Frequency of the power source: 20 kHz
→ Wavelength: 15 km ≫ Cell size

Distributed constant lines

⇩

Unit lumped element of Δx $(\ll \lambda)$
→ The line is represented by the longitudinal connection of the element



Equivalent circuit network

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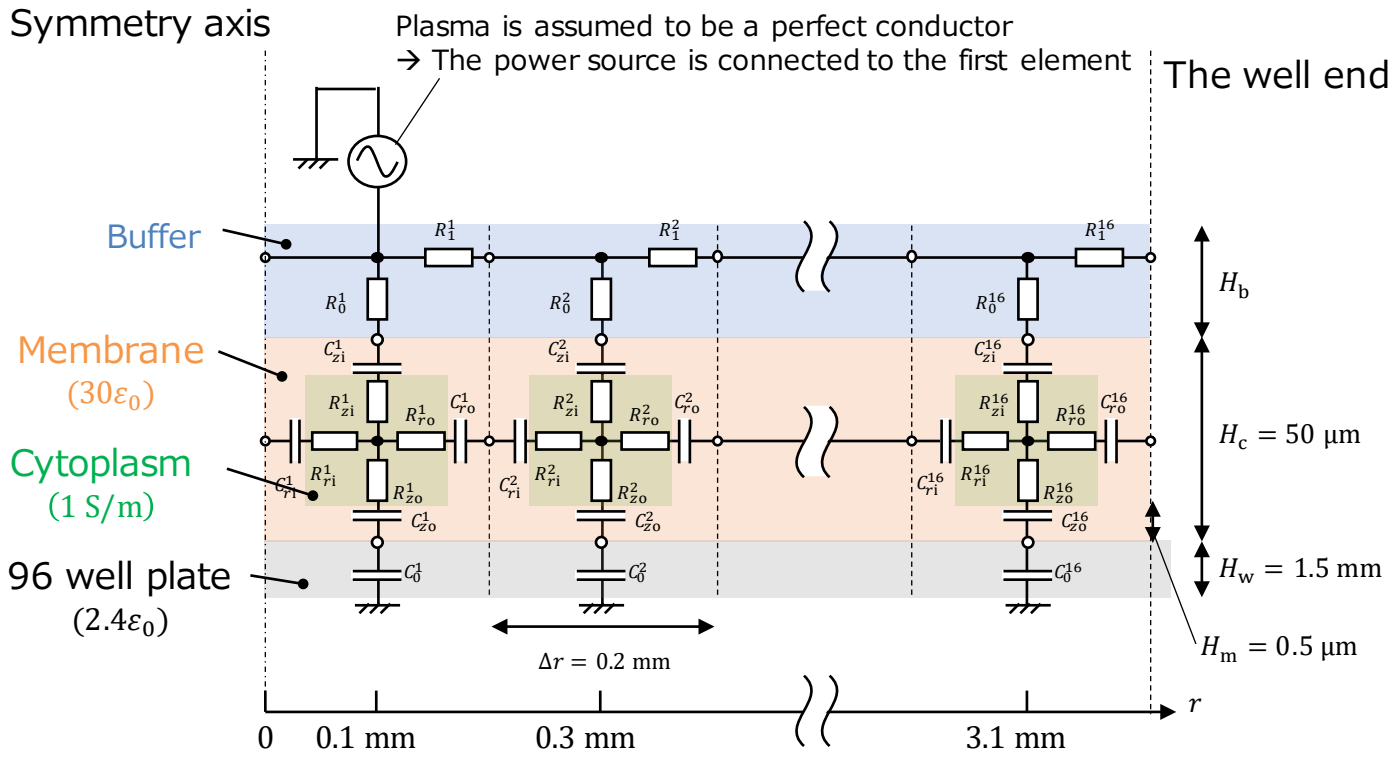
The modeled circuit



Steady state analysis by Ltspice
Voltage and current of each element is obtained
→ Electric field and current density on the cell are analyzed

Analysis condition can be changed
by the circuit parameter of each element

Comparison with transfection efficiency

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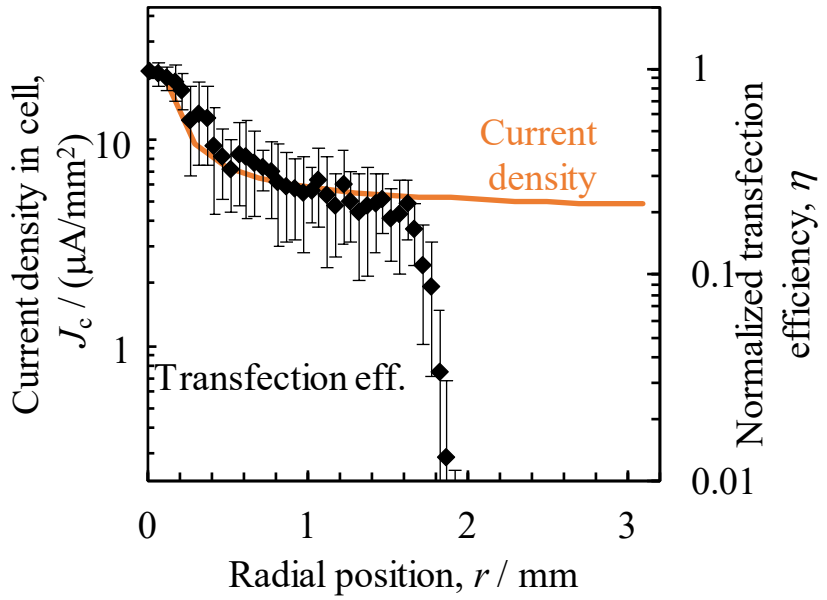
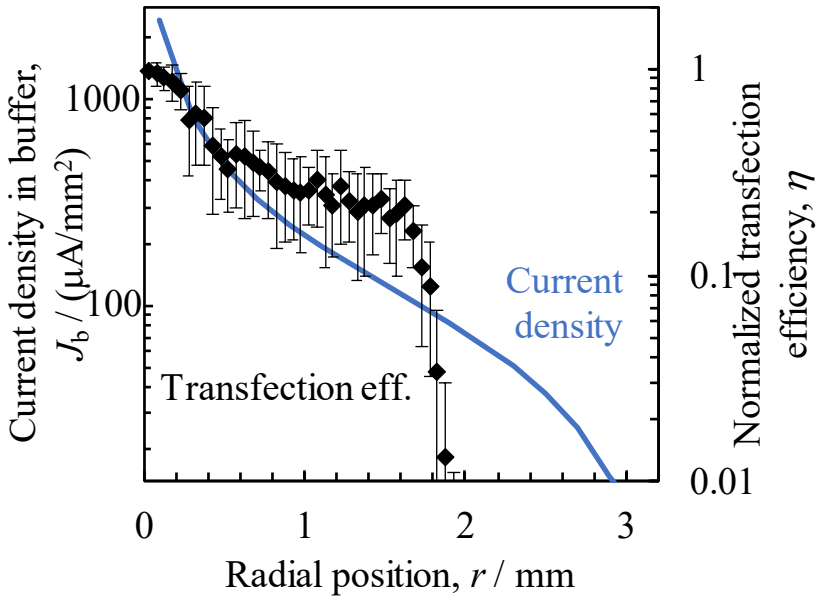
Membrane



Buffer



Current density on the membrane shows
similar radial distribution to the transfection efficiency ($r < 1.6$ mm)

⇩

- Suggests contribution of electrical factors to the membrane
- Current density or electric field? → Need to check absolute the values.

Absolute values

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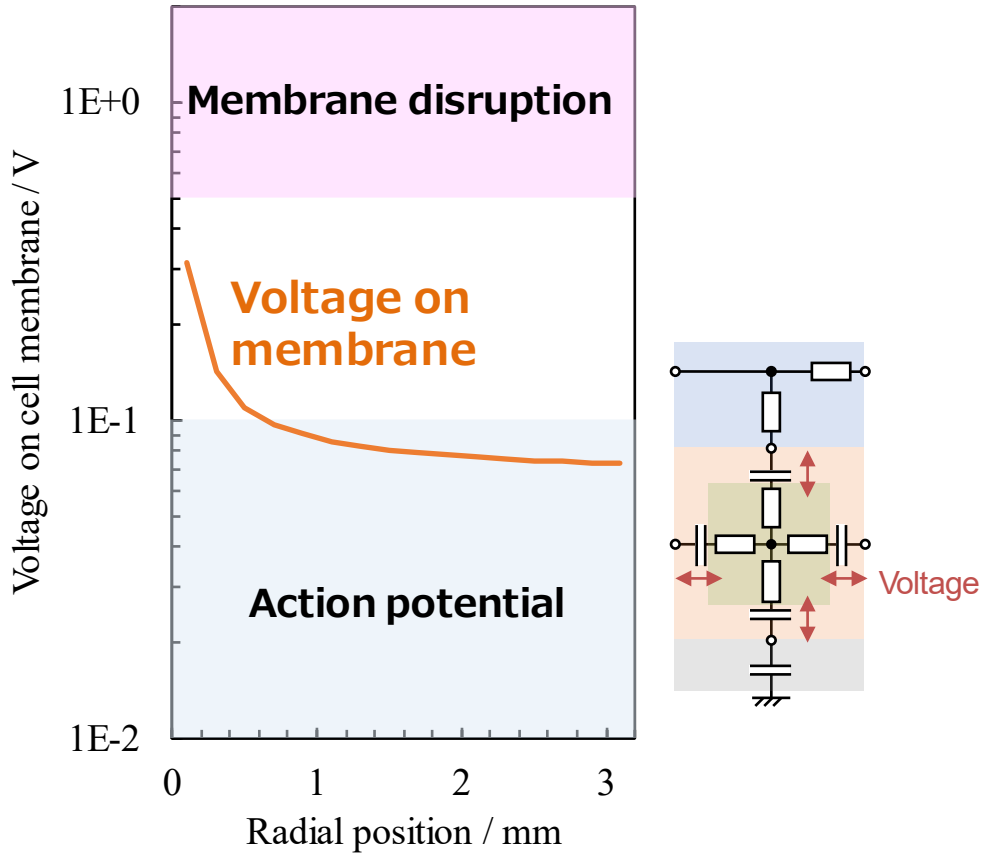
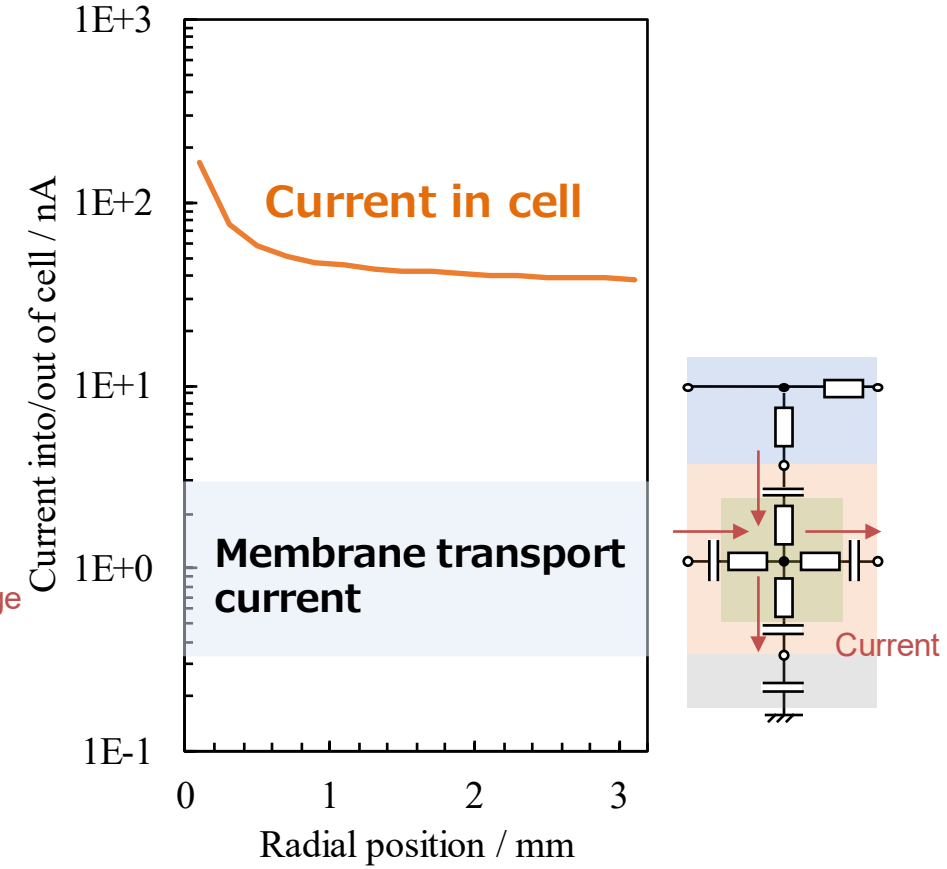
Voltage (E. field × membrane thickness)



Plasma contribution
≈ ordinary cellular action

Current (Current den. × cell surface)



Plasma contribution
≫ ordinary cellular action

[4] Y. Kido *et al*, PLoS ONE, **16,** e0245654 (2021)

Suggests that the main electrical factor is the current

Changing Cell Density

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Plasma

Results

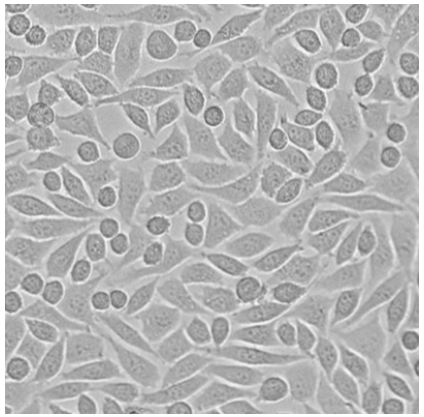
Before plasma treatment

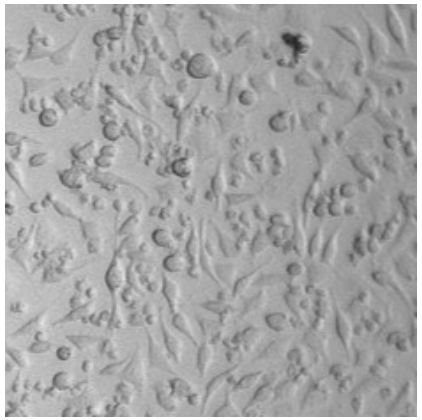
After plasma treatment

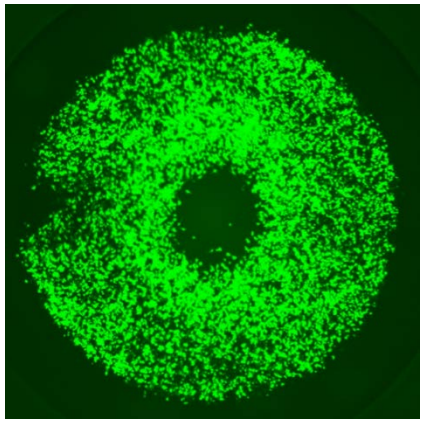
Plasmid Expression **Green**

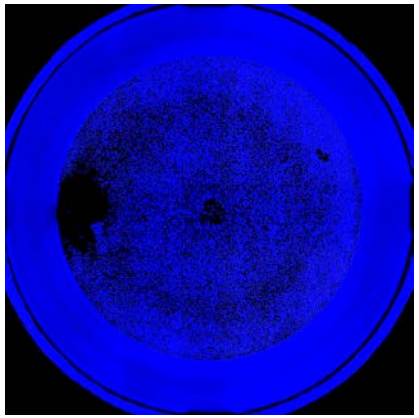
Surviving Cells **Blue**

Cell density 100 %

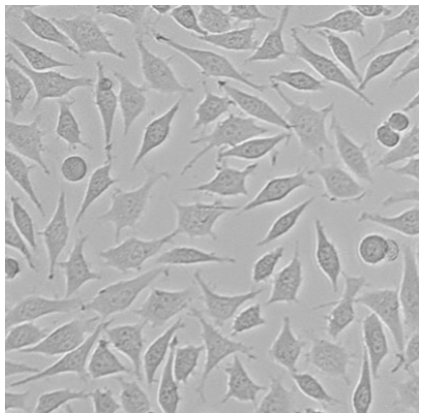


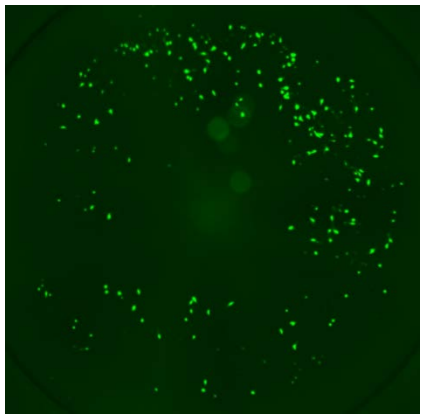


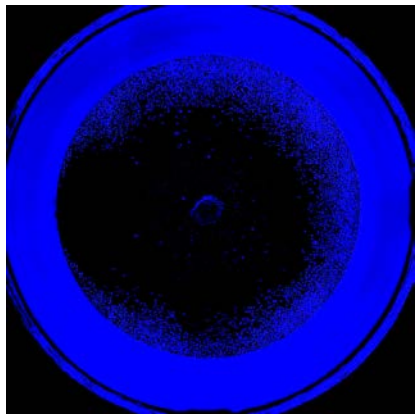




Cell density 50 %







Model

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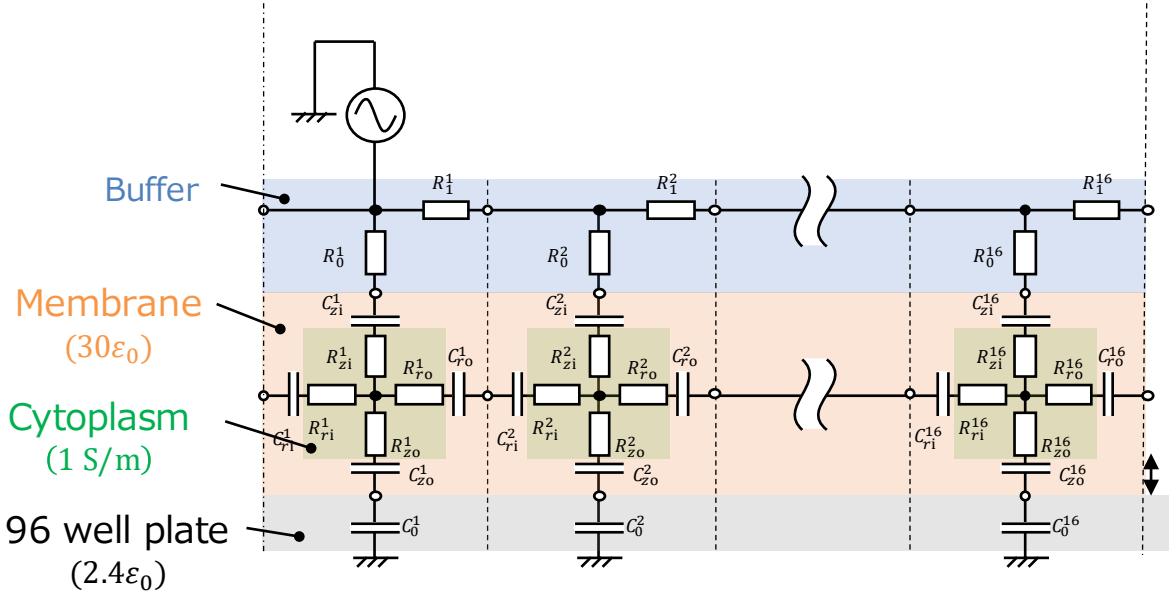
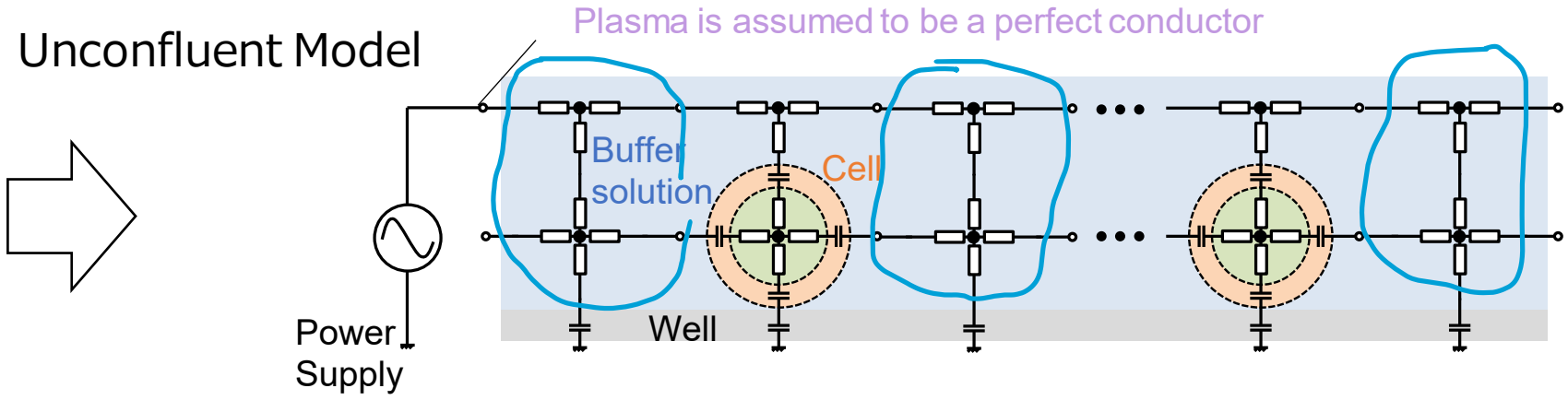
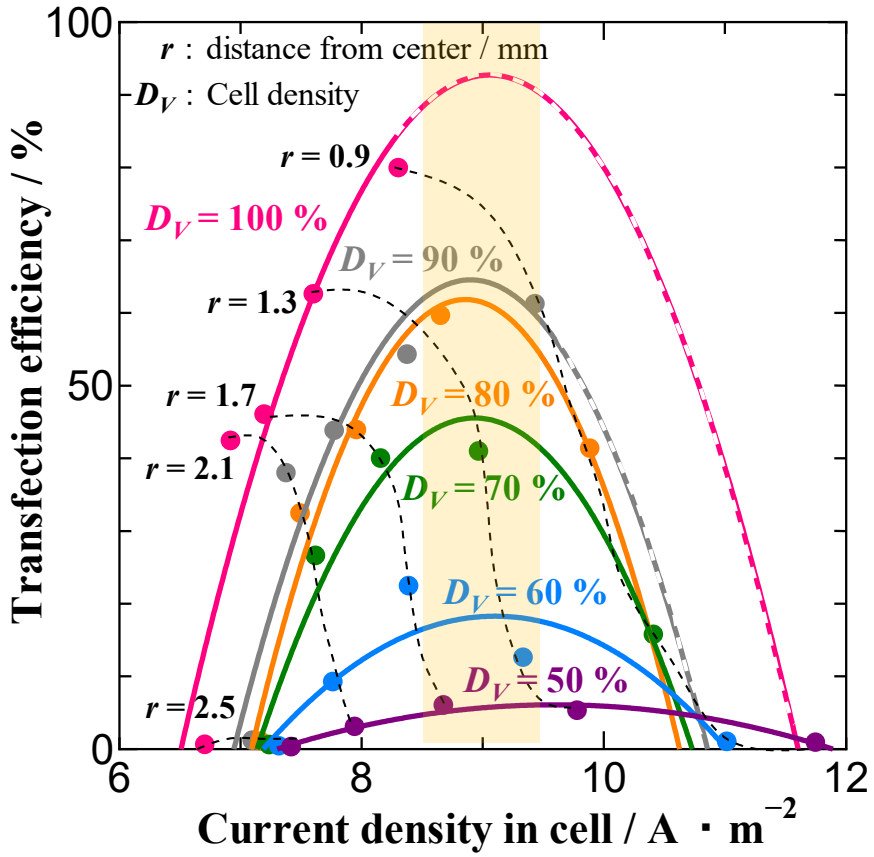
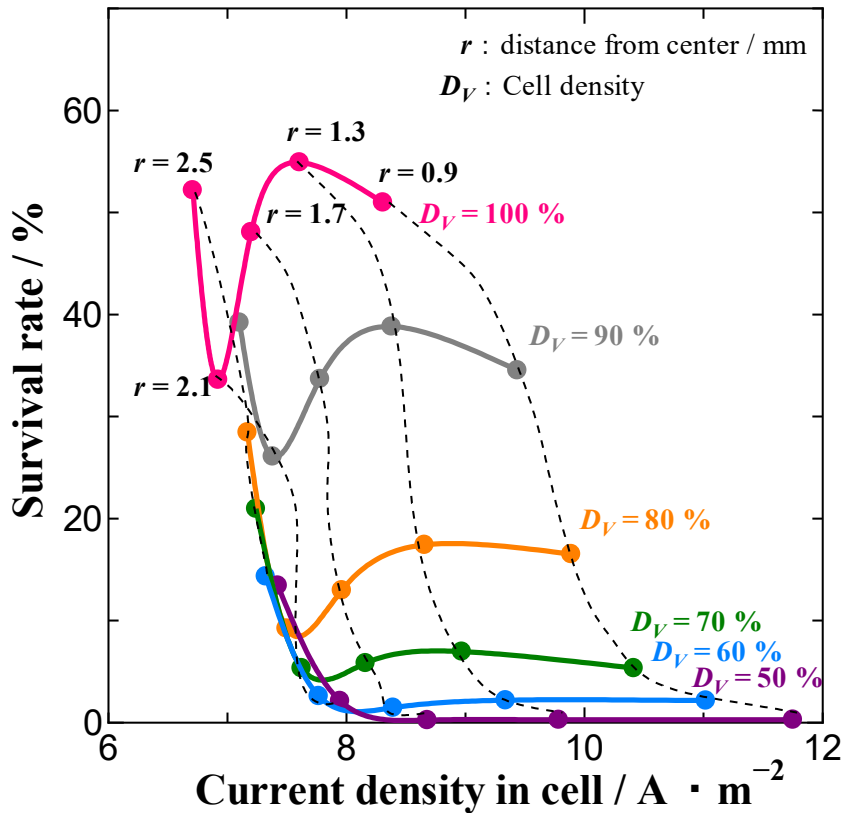
Confluent Model



Unconfluent Model



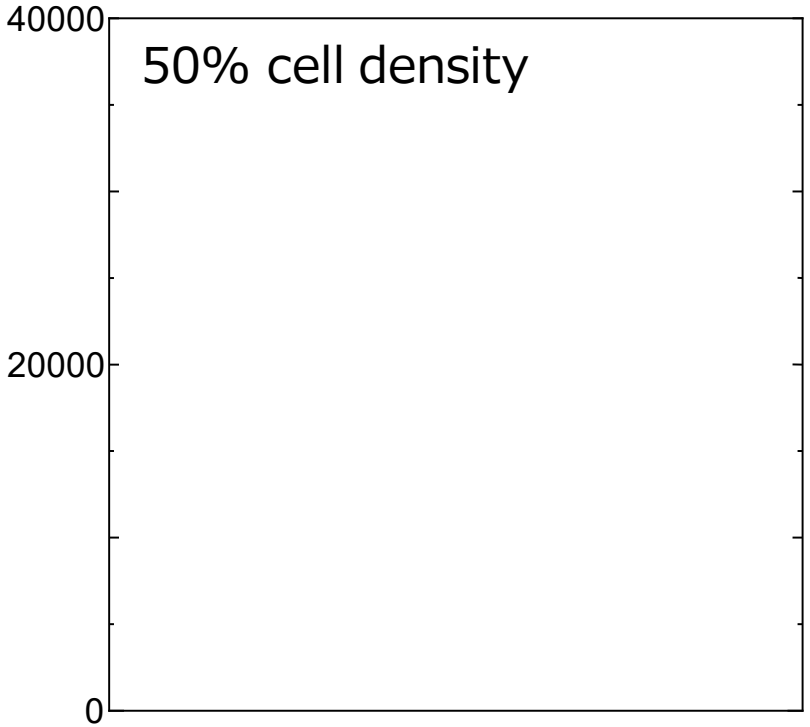


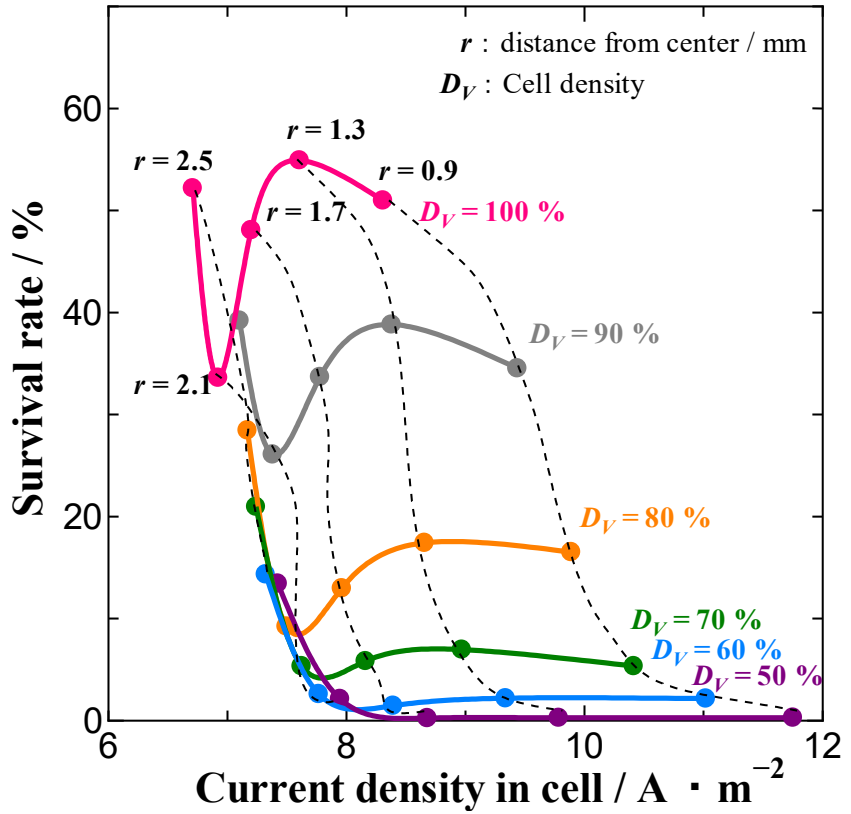


Optimum Current Density is Constant

Cell viability is critically affected by cell density.

- The optimum value of intracellular current density at which transfection efficiency is high is almost constant regardless of cell density
- The maximum transfection efficiency value differs according to the cell density: higher transfection efficiency is obtained at the higher cell density





Optimum Current Density is Constant

Cell viability is critically affected by cell density.

By scavenging ROS, cell viability is improved.

ROS may cause cell death.

At the center,
plasma causes cell death.

- The optimum value of intracellular current density at which transfection efficiency is high is almost constant regardless of cell density
- The maximum transfection efficiency value differs according to the cell density: higher transfection efficiency is obtained at the higher cell density

Contents of the Talk

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1. Plasma Gene/Molecular Introduction
2. The Mechanism of Introduction
   - Current or Field?
3. The Random Genome Integration-free
   - Spontaneous Uptake induced by complex of stimuli
4. The Applications and Future Tasks of the Plasma Gene/Molecular Introduction
5. Growth Acceleration of Fish using Plasma

Random Genome Integration with Various Gene Introduction Methods

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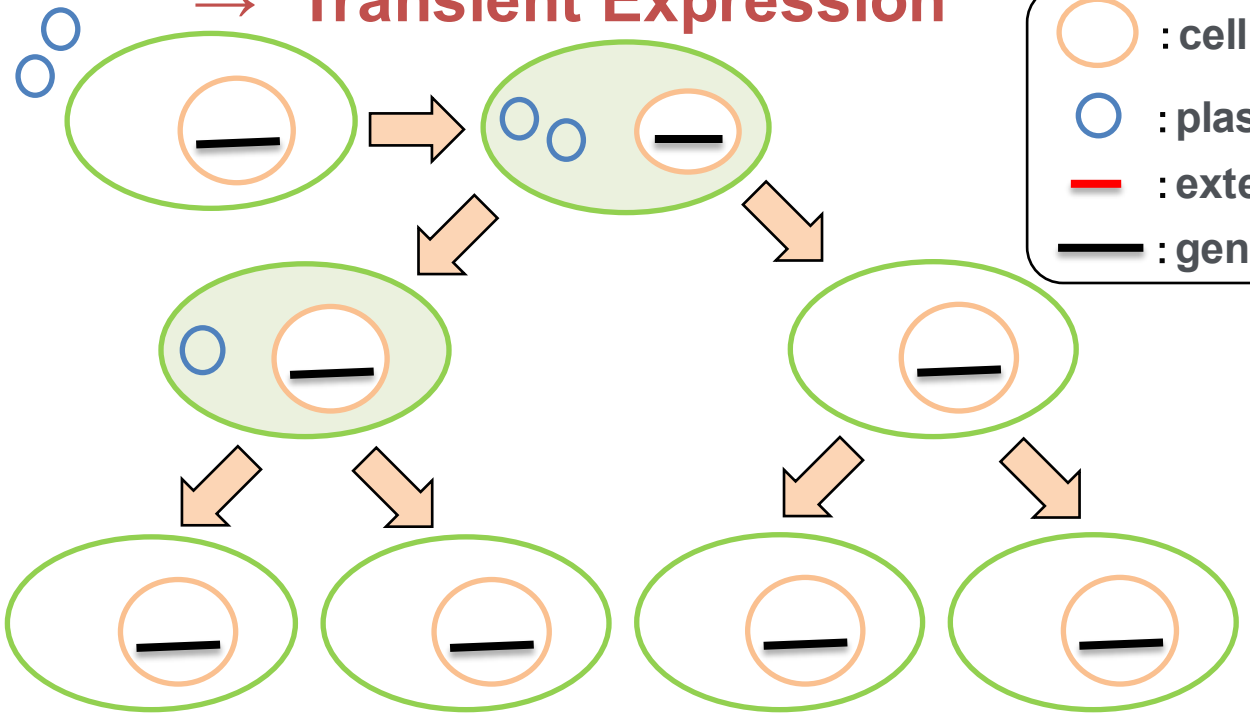
Spontaneous Uptake (Endocytosis) → Transient Expression

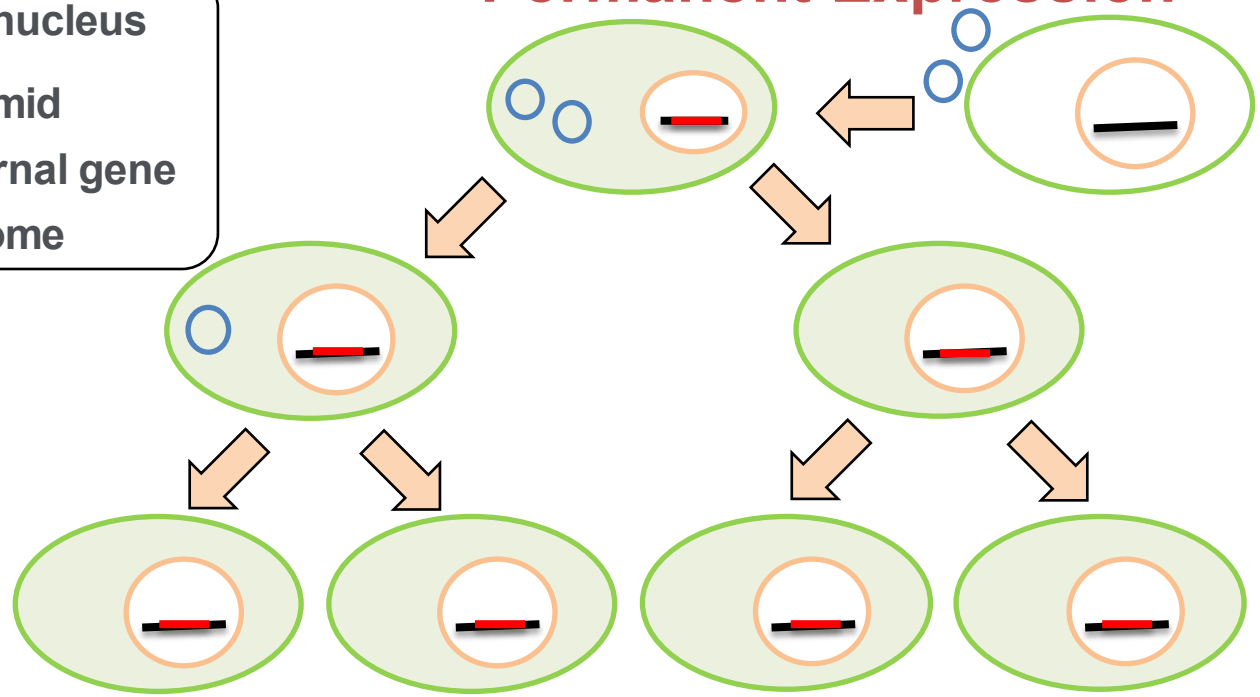


: cell nucleus
: plasmid
: external gene
: genome

External gene is not integrated into the genome, at some point it disappears by autophagy.

Ideal for genetic and regenerative medicine.

Legacy Methods → Permanent Expression



External genetic information is eventually integrated into the genome, perpetuating its expression.

Not applicable to genetic and regenerative medicine

Random Genome Integration with Various Gene Introduction Methods

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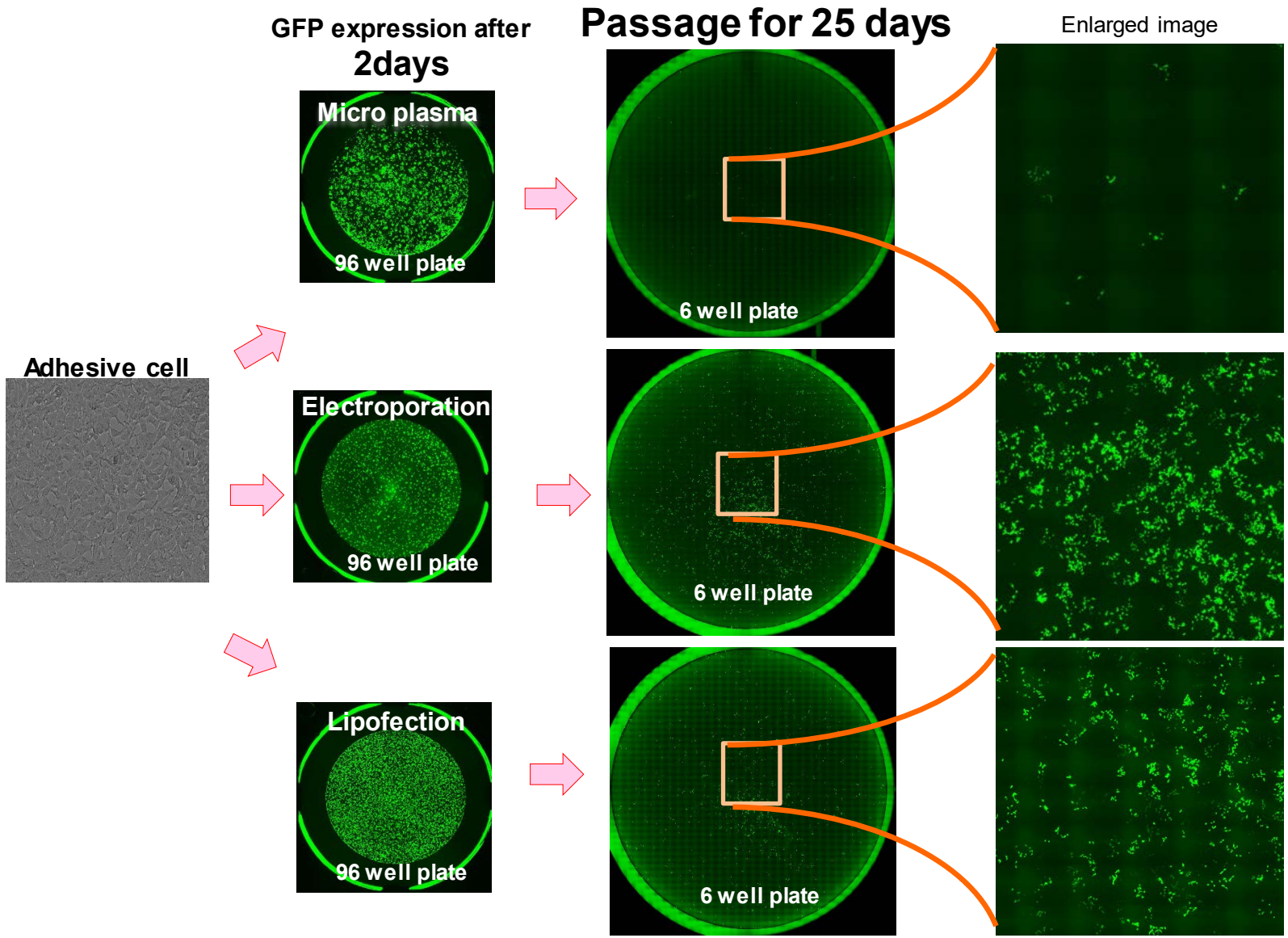
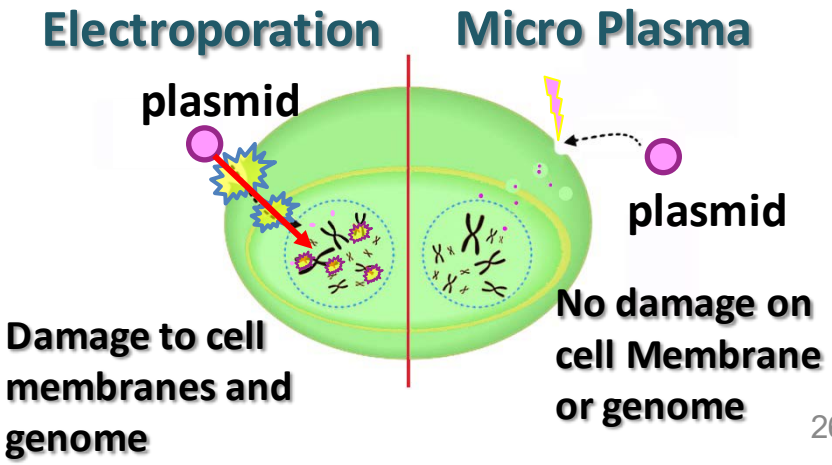


Micro-Plasma method introduces external molecules and genes **without Random Genome Integration.**

Legacy methods can not achieve **"Random Genome Integration Free."**

Micro plasma gene/molecular introduction is suitable for gene-therapy with genome editing.



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Applications: for Regenerative Medicine

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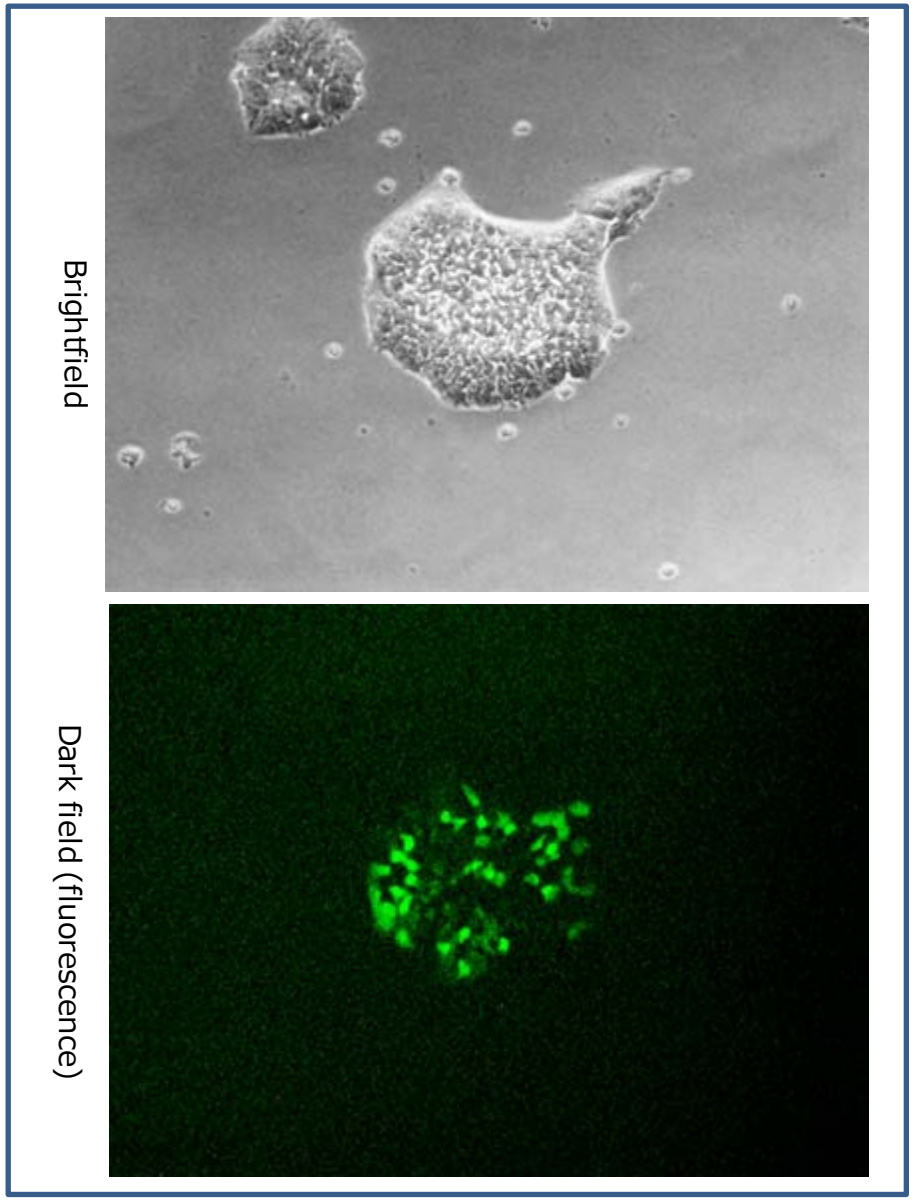
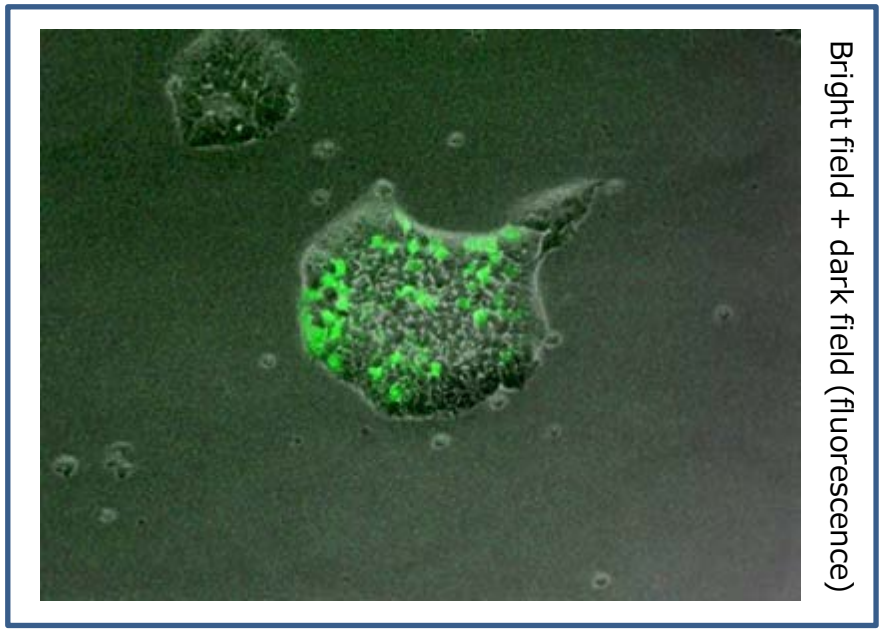


Geme introduction into iPS-cells



Applications: for Regenerative Medicine

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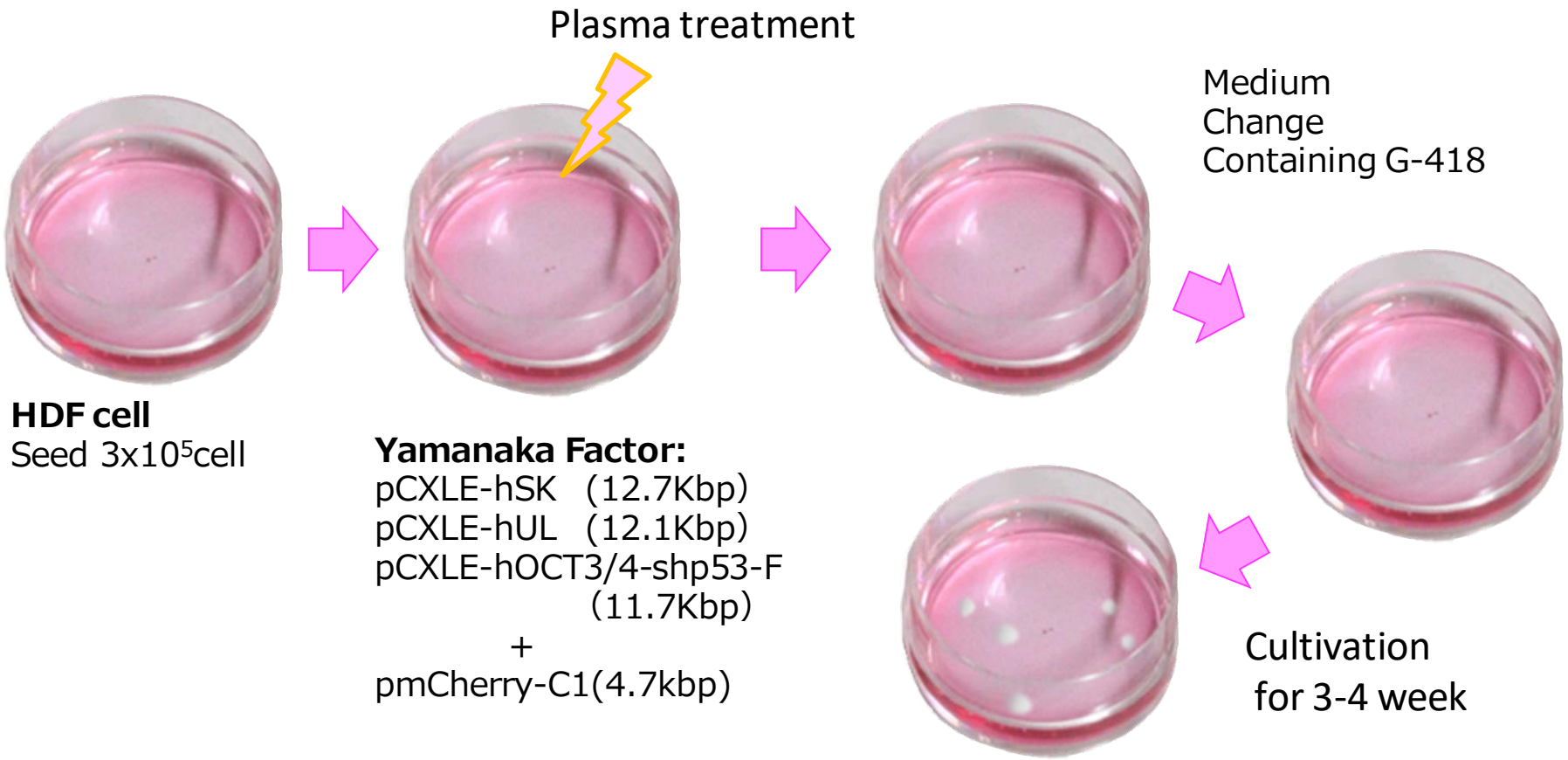
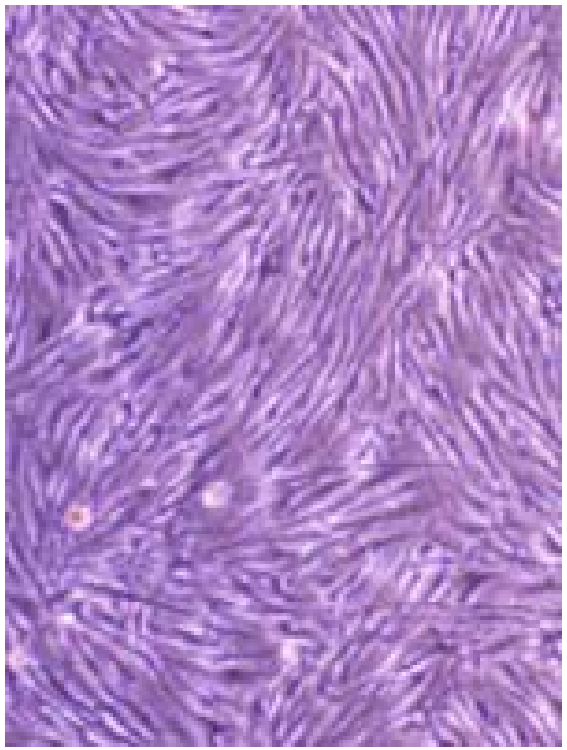
iPS-cell establishment



☆IPS like cells can be formed in about one month after only medium exchange

Applications: for Regenerative Medicine

iPS-cell establishment



Yamanaka Factor
+
Tet1 gene

With plasma irradiation

Human Dendritic Fibroblast Cell

Human iPS Cell

Applications: for Genetic Medicine

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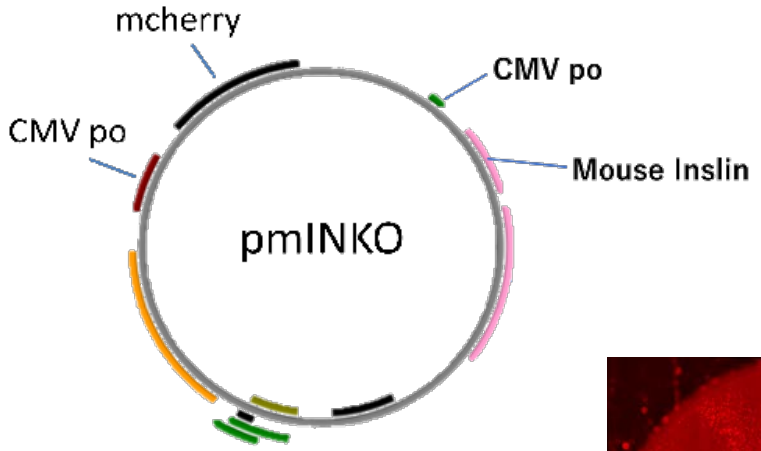
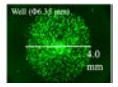
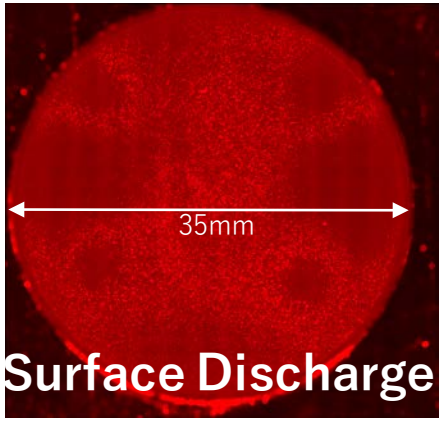
Cell Therapy：
Challenges in the treatment of type I diabetes mellitus

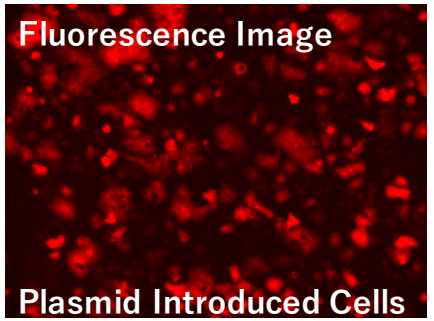
pmINKO plasmid:
Introduced cell produces insulin and express red fluorescence.

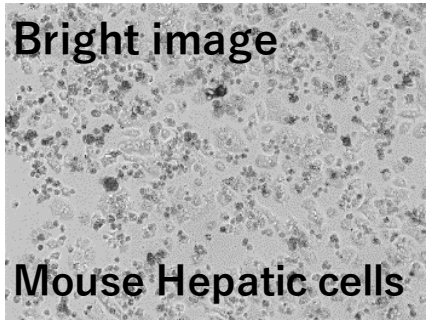


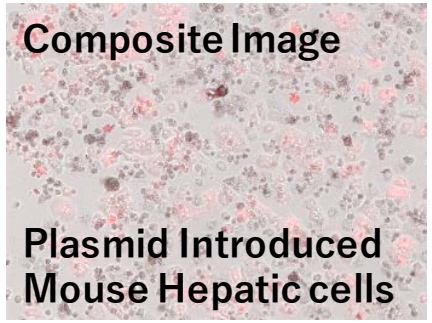


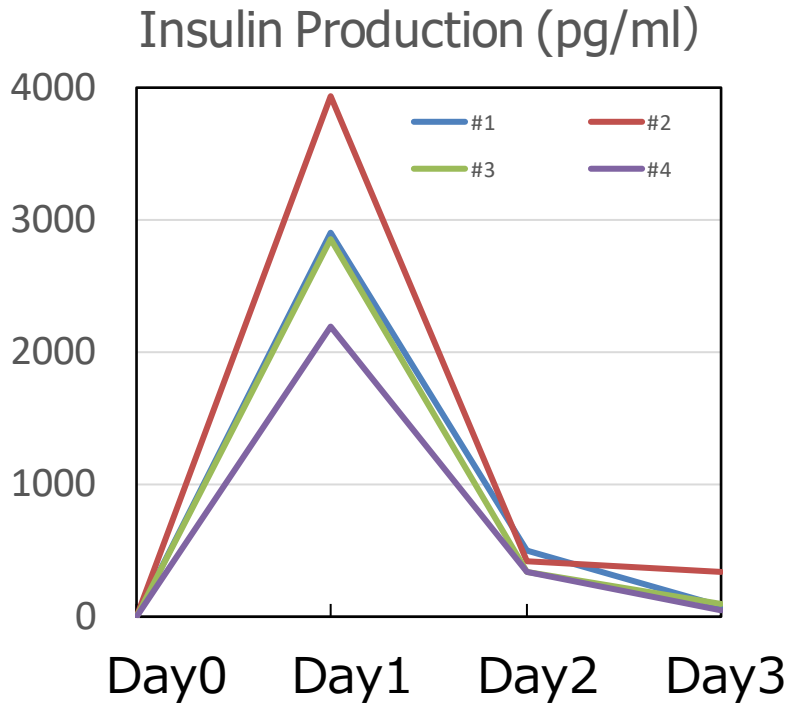
microplasma











It has been demonstrated that the plasma method can create transformed cells by introducing genes into primary cells.

Applications: for Plant Breeding

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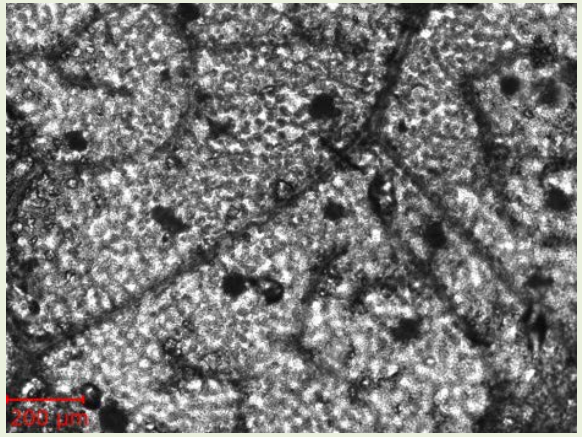
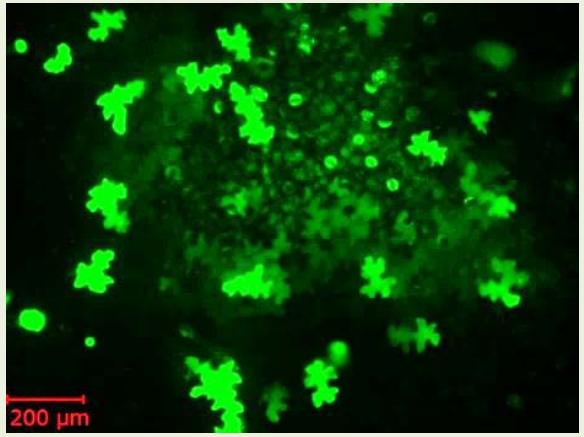
Plasma method enables gene introduction into plant cells.

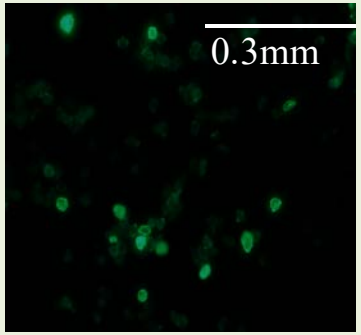
Fluorescent molecule introduction into plant cells

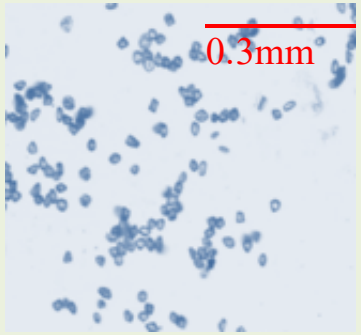




The mechanism of molecular introduction into plant cell will be presented in the **poster session 3 (P3-48) on Thursday.**

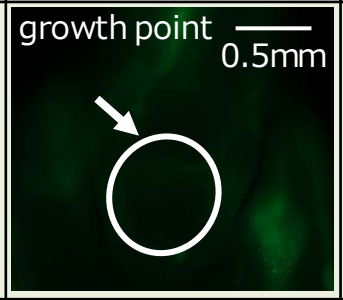
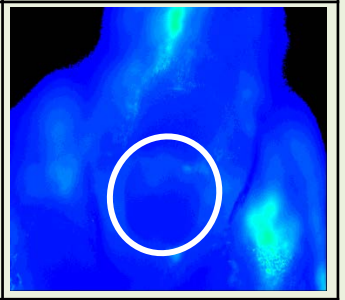
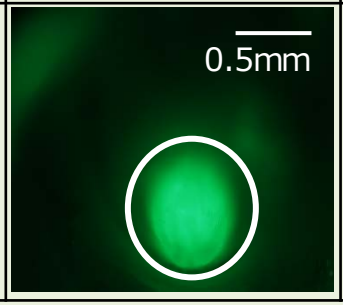
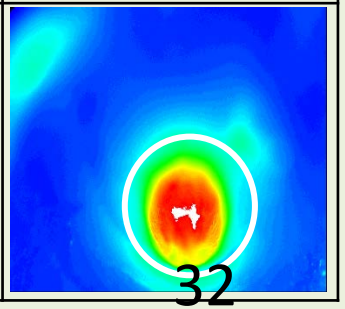
Fluorescent molecule introduction into kiwifruit pollens





Fluorescent molecule introduction into growth point

Growth point of barley

	Darkfield	Color mapping
Control		
Plasma		

Applications: for Fish Breeding

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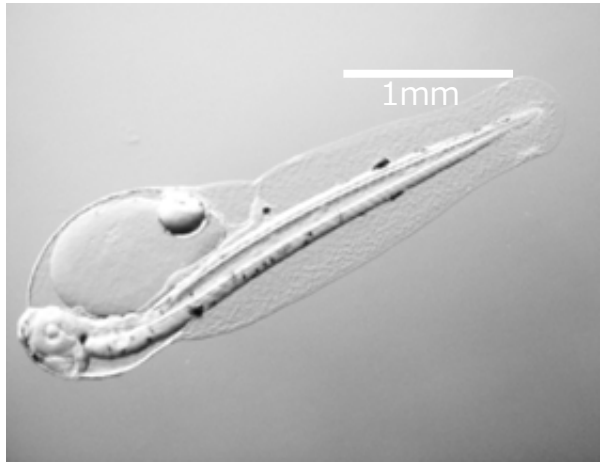
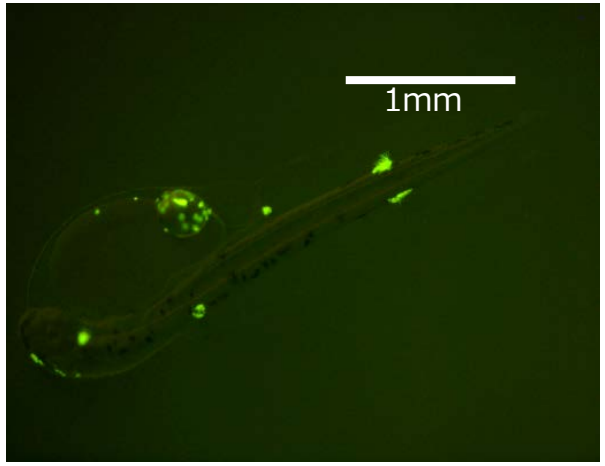
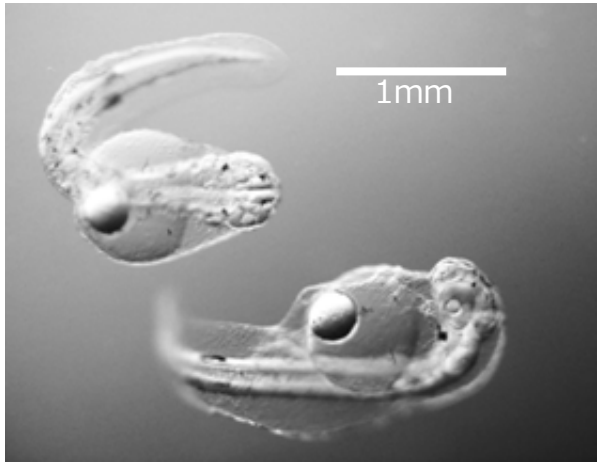
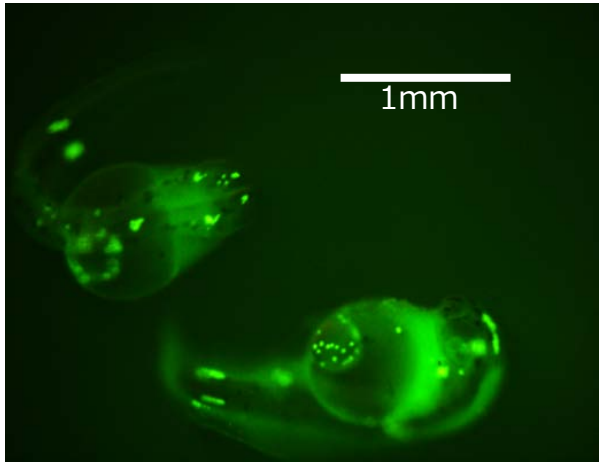
Enables introduction of macromolecular into fish eggs.

	Hatched fish	Fluorescence Image
Control		
Plasma Treated		

Development and Commercialization

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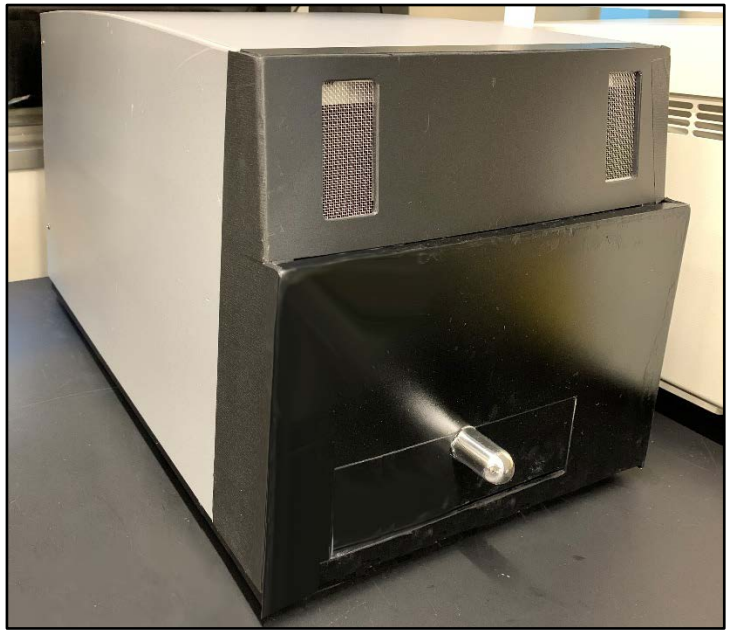
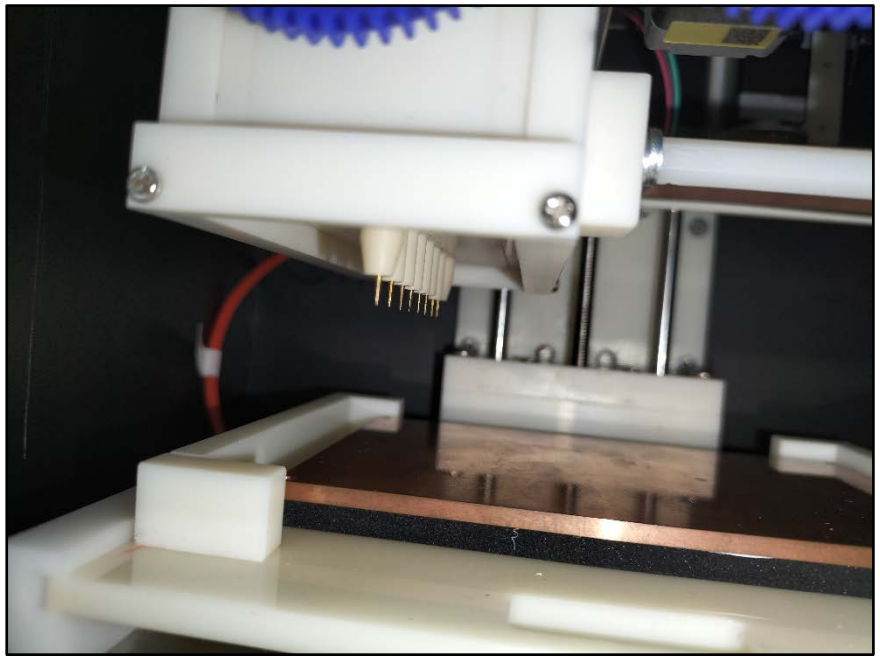
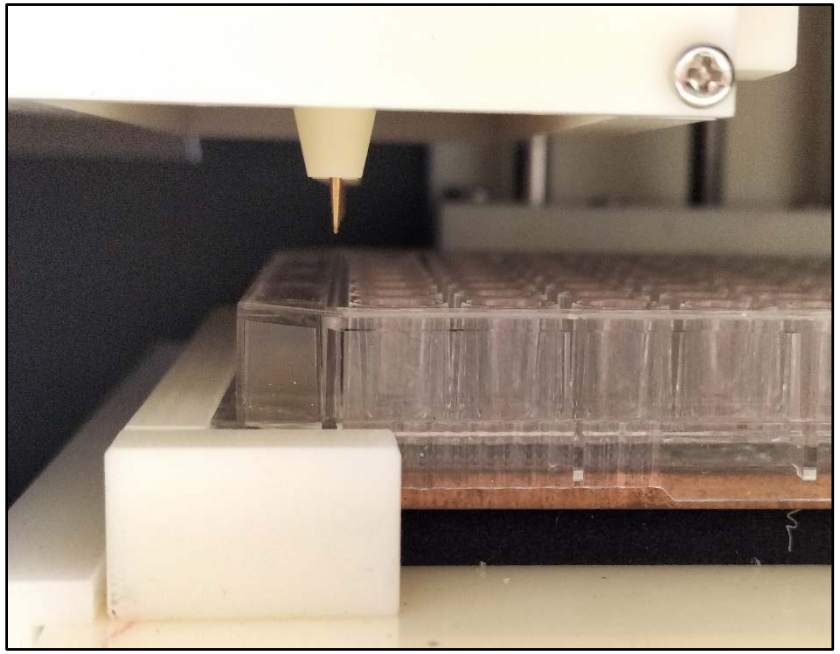
Pre-Production Model is Ready.
The device for research will be on the market before the next Summer.







Future Tasks

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Scientific Subjects

- Diagnostics: Plasma parameters are unknown
- Modelling: Plasma should be expressed not as perfect conductor in the circuit model
- Mechanism: What happens inside the cell?

Application Subjects

- Mass Treatment: Surface Discharge
- Confirm Non-invasiveness, Random-Integration-Free in detail by specialists
- To be well-know the technology in the medical and bio fields

Surface Discharge Method for Mass Treatment

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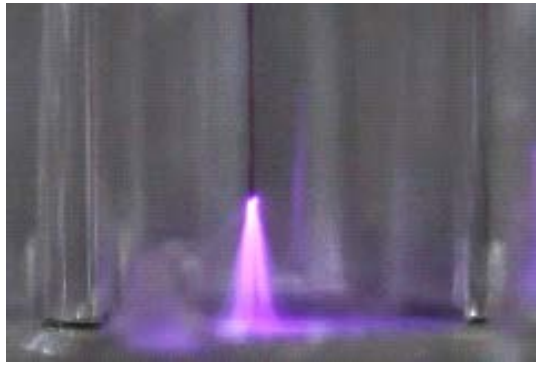
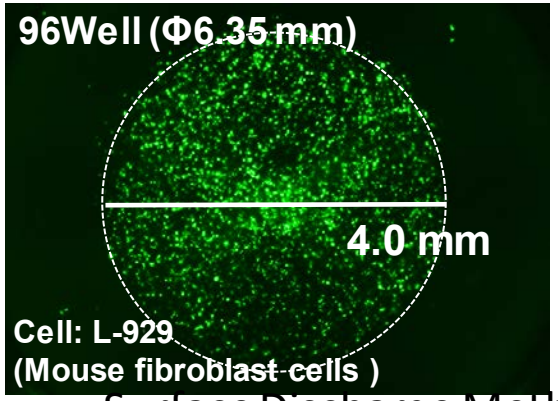
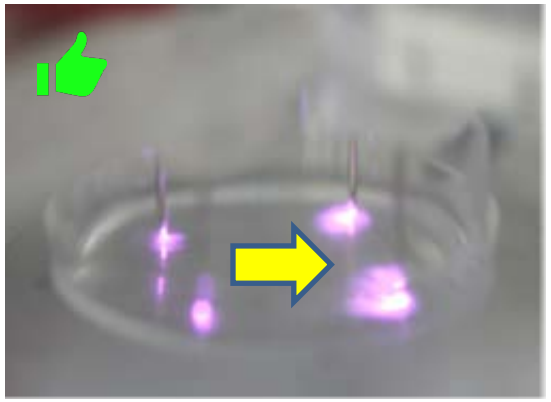
Micro plasma

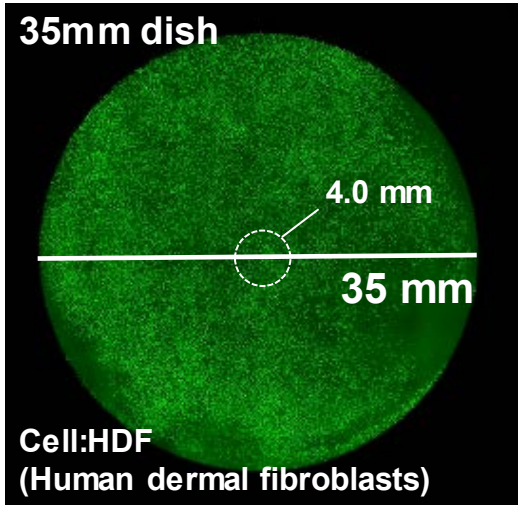


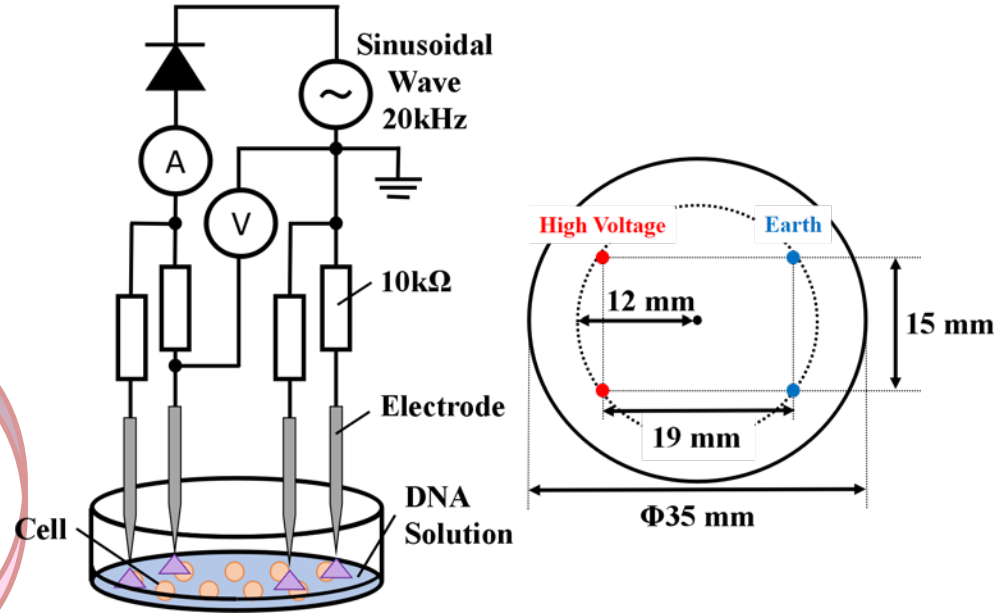


Surface Discharge Method

Surface Discharge

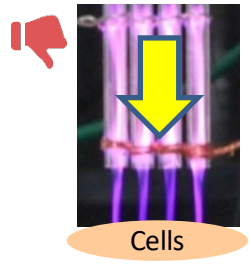


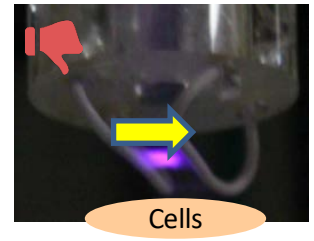


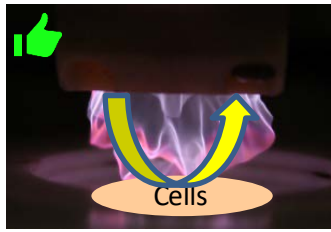


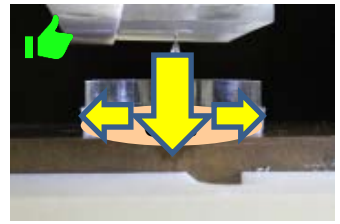
Voltage : 4.0 kV, Irradiation Time : 5 msec
Frequency of supply : 20 kHz, Gap Length : 800 μm

T. Hiramatsu *et al. Jpn. J. Appl. Phys.* **58**, SEEG05 (2019)









Current passes through cells

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Growth Acceleration of Fish with Plasma-treated Air-Supplied Water (PAW)

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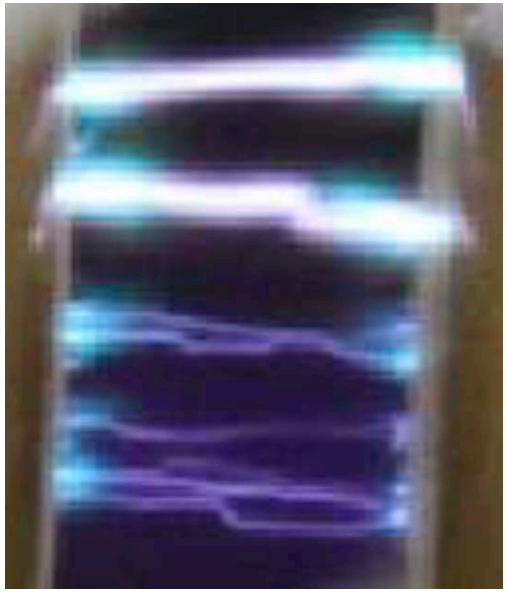
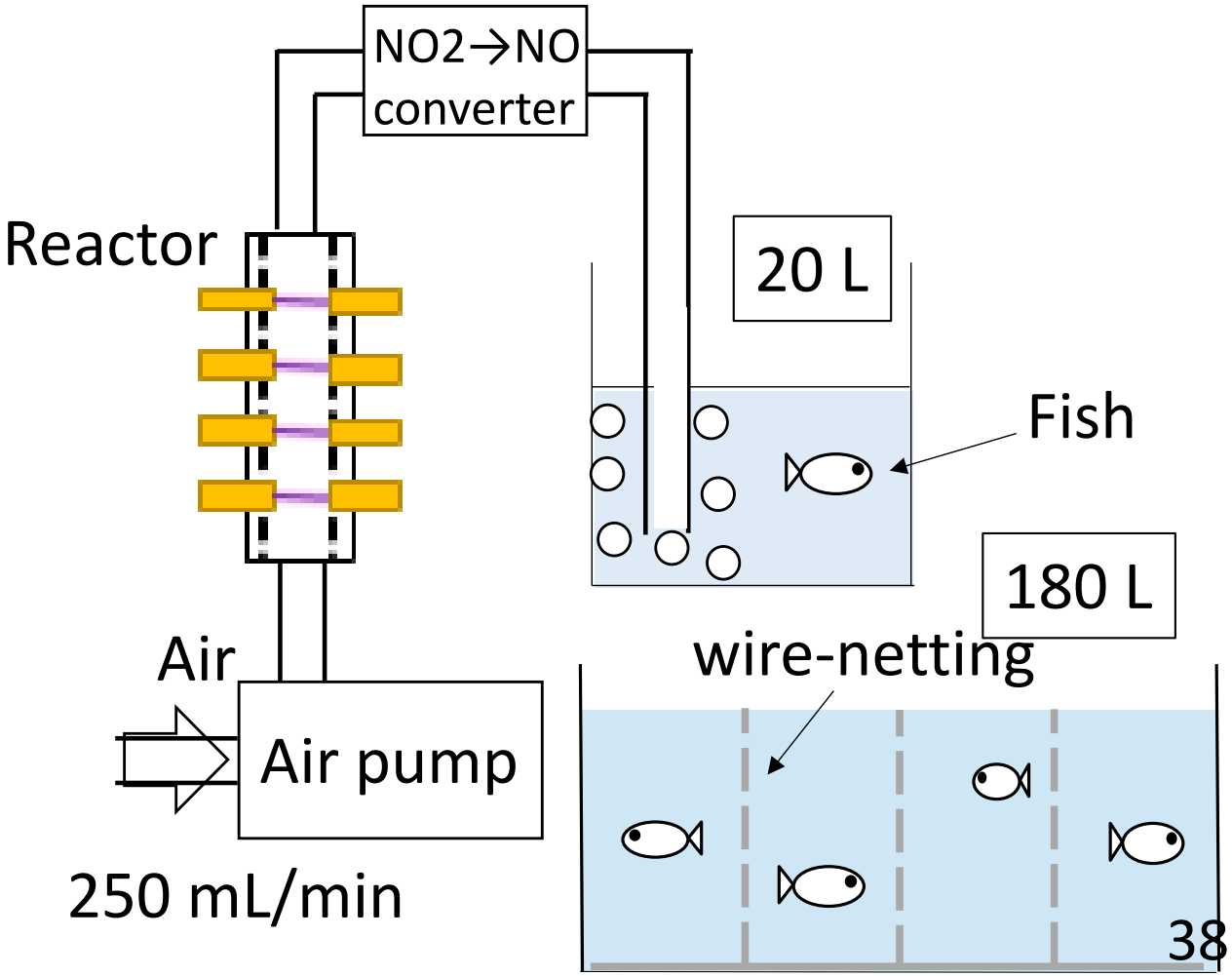


Treatment time is

Only less than 10min. each day.

$$2NO_2 + H_2O \rightarrow HNO_3 + HNO_2$$



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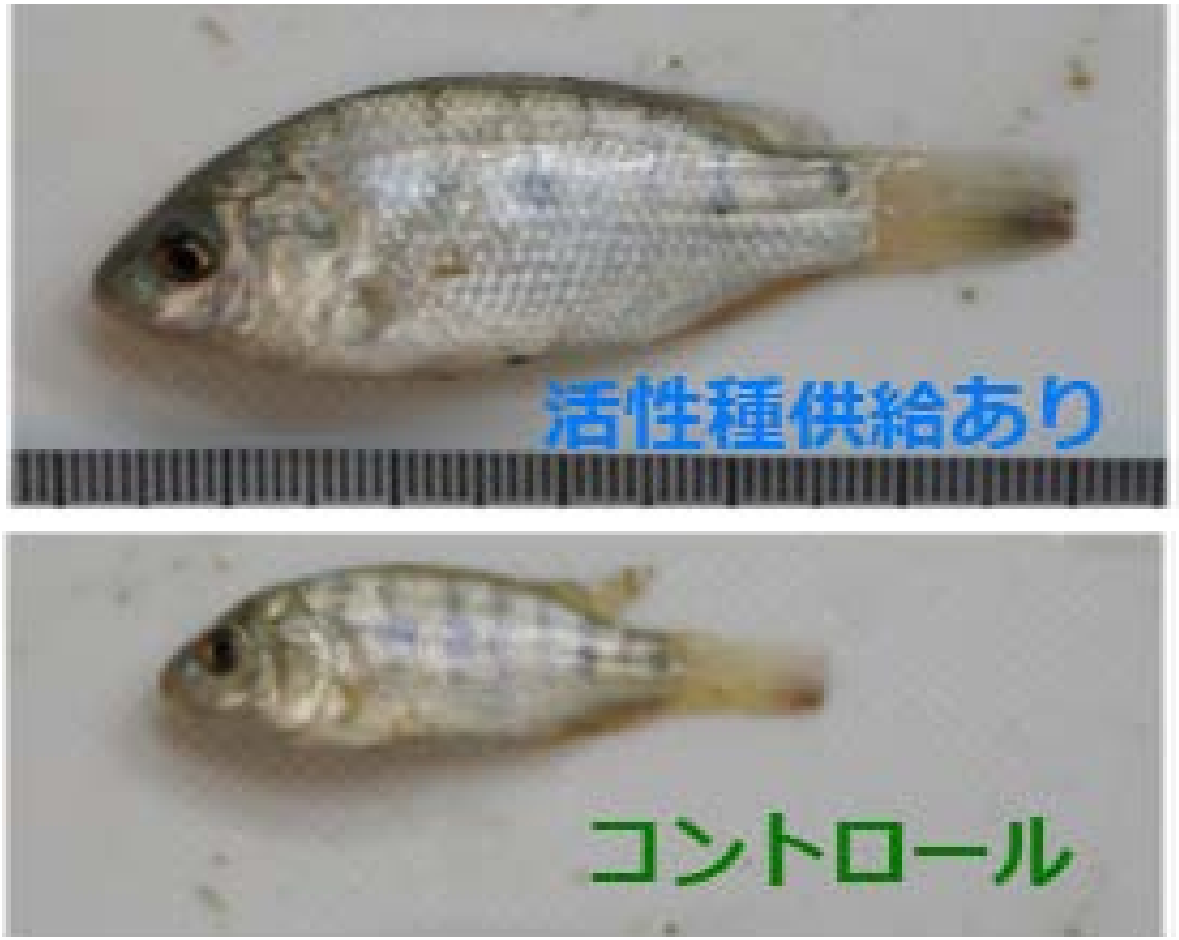
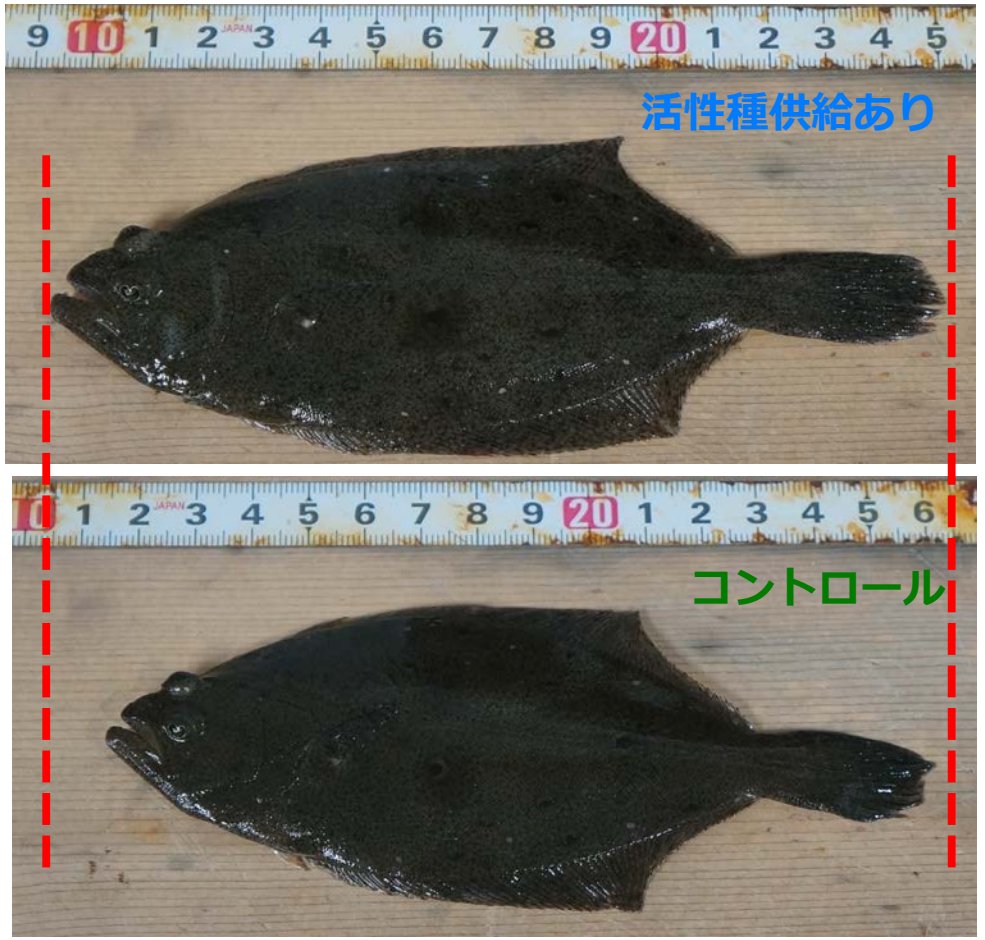
In PAW

Control



Nile Tilapia 20 weeks later (or more)



Flounder 6 weeks later

Y. Ikeda et al. *Free Radical Research* **57**(1), 21–29 (2023)

instead of Conclusion

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1st Type : Energy Conversion/Generation

Plasma specialists

Generate something

Light Sources

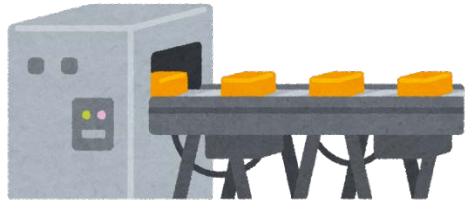
Fusion

2nd Type : Material Conversion

with Background of Physics/Chemistry

Produce something

Change the structure



Plasma Deposition

Plasma Etching

Plasma Torch

3rd Type : Process Trigger

Far from plasma unknown with fear

Indirectly induce something

Plasma activated X

Plasma assisted X

cf. Germination