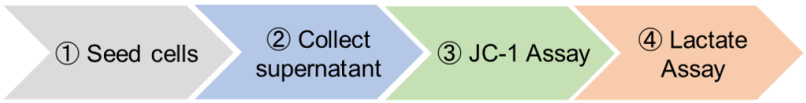
Glycolysis/JC-1 MitoMP Assay Kit

G272 Product Manual

-General Information

Glycolysis is one of the main metabolic pathways in cells, and lactate is a marker for monitoring glycolytic function. Mitochondria are one of the most important organelles in energy production for living cells, and mitochondrial membrane potential is widely used as a marker of mitochondrial function. Some papers have suggested that senescent cells, induced mitochondrial dysfunction, enhance glycolysis[1], and if glycolysis is suppressed in cancer cells that are strongly dependent on glycolysis, their viability is maintained by enhancing mitochondrial function[2]. It is expected that confirming the functions of both glycolysis and mitochondria will play a key part in the development of therapeutic strategies for senescence, cancer, and various diseases.

The Glycolysis/JC-1 MitoMP Assay Kit enables analysis of both lactate production (Lactate Assay) and mitochondrial membrane potential (JC-1 Assay). The kit includes all the reagents required for these evaluations.



Experimental flow

-Kit Contents

Dye Mixture (Blue cap)	× 1	Use for Lactate Assay
Lactate Standard (10 mmol/l) (Blue cap)	150 µl × 1	
LDH Solution (Green cap)	12 µl × 1	
Lactate Assay Buffer	5.5 ml × 1	
Reconstitution Buffer (White cap)	550 µl × 1	
JC-1 Dye (Red cap)	× 1	Use for JC-1 Assay
Imaging Buffer (10 ×) (Red cap)	6 ml × 1	

Note: This kit includes the reagents corresponding to 48 wells of a 96-well microplate. The number of samples that can be evaluated with this kit is posted on the product web page.

Note: Evaluation in the Lactate Assay is performed by relative evaluation using absorbance values.

-Storage Conditions

Store at 0-5 ° C

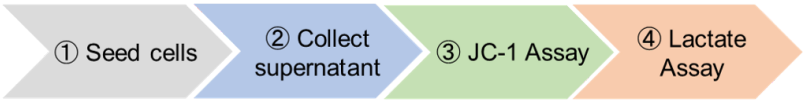
-Required Equipment and Materials

- Microplate reader (450 nm filter)
- Fluorescence microscope, fluorescence microplate reader
- 96-well microplate, 96-well black microplate (clear bottom)
- Incubator (37 ° C)
- 20-200 µl multichannel pipette
- 100-1000 µl, 20-200 µl, 2-20 µl micropipettes
- Dimethyl sulfoxide (DMSO)

－1.5-ml microtube
－Conical tube

-Precautions

・Equilibrate reagents to room temperature prior to use.
・Briefly centrifuge the tubes before opening to ensure the content is at the bottom.
・Pipette the LDH Solution (used for the Lactate Assay) before use to ensure the enzyme solution is homogenous.
・Since the enzymatic reaction starts immediately after adding Lactate working solution to a well, use a multichannel pipette to minimize the experimental error from time lag in pipetting.
・Please refer to Table 1 for suitable fluorescence wavelengths for the JC-1 Assay.
・Analysis of samples in triplicate is recommended for accuracy.
・The Dye Mixture is stored in a glass bottle with an aluminum cap. Handle with caution, and wear gloves.
・Please perform the JC-1 Assay first (refer to flowchart below).



Experimental flow

Table 1. Recommended filter settings for the JC-1 Assay

Fluorescence microplate reader	Confocal microscope	Fluorescence microscope
Green Excitation 480–490 nm Emission 525–545 nm Red Excitation 530–540 nm Emission 585–605 nm	Green Excitation 488 nm Emission 500–550 nm Red Excitation 561 nm Emission 560–610 nm	Green GFP or FITC filter Red Cy3 or TRITC filter

-Preparation of Solutions and Measurement (Lactate Assay)

Preparation of Dye Mixture stock solution

Add all Reconstitution Buffer (White cap) to a Dye Mixture vial. Close the cap and dissolve the contents completely.

Note: Transfer the Dye Mixture stock solution to the vial containing the Reconstitution Buffer and store it at 0-5 ° C with protection from light. Dye Mixture stock solution is stable for 4 months under these conditions.

Preparation of Lactate working solution

(1) Add Dye Mixture stock solution to a conical tube and dilute it with Lactate Assay Buffer.
(2) Add LDH Solution (Green cap) to the solution prepared in step (1).

Note: Refer to Table 2 for the amounts to be mixed.

Note: Lactate working solution is light sensitive. Prepare the solution just before use and protect it from light by covering it with aluminum foil. Please use up the Lactate working solution within that day.

Table 2. Examples of Lactate working solution preparation

	for 24 wells	for 48 wells
Dye Mixture stock solution	250 µl	500 µl
Lactate Assay Buffer	2.25 ml	4.5 ml
LDH Solution (Green cap)	5 µl	10 µl

Sample preparation

Prepare cell culture supernatant sample.

Note: Please prepare samples diluted with double-deionized H_2O (ddH_2O) so that the absorbance is lower than 1 mmol/l Lactate standard solution (see Figure 1).

Note: If a medium contains serum, read the blank absorbance (serum-containing medium) as a background control and subtract its value from the absorbance of each

sample.

Note: The required amount of sample is 20 µl for each well.

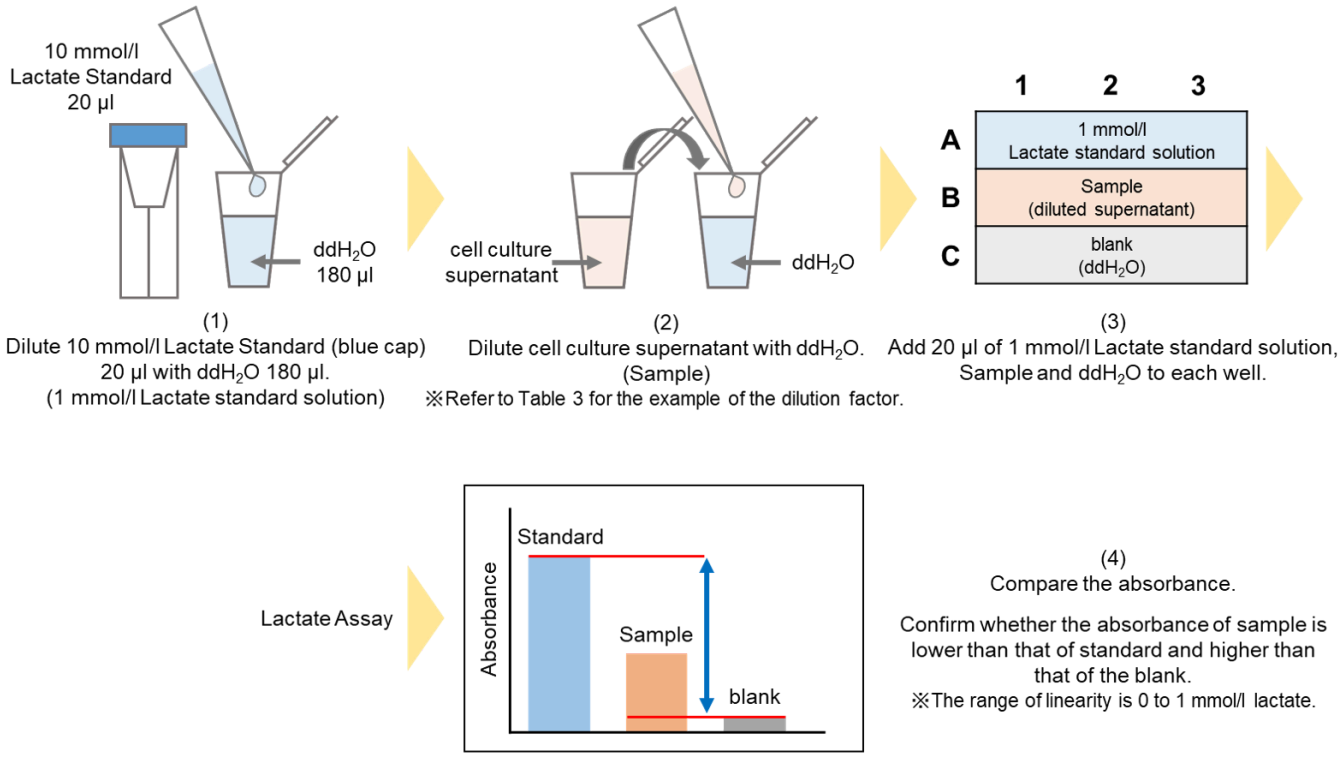
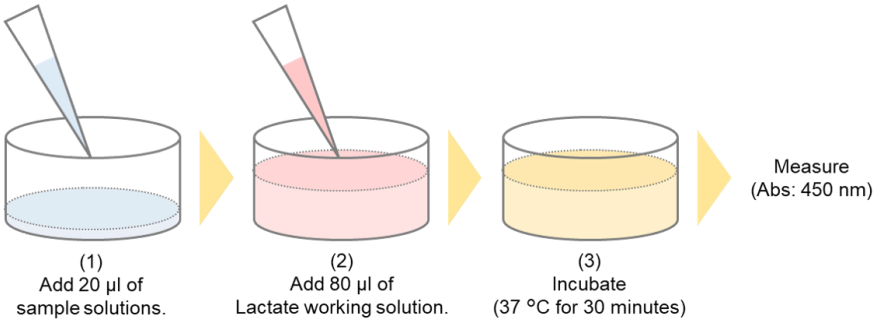


Figure 1. Method for dilution of samples for Lactate Assay

Table 3. Examples of dilution

		Inhibitor treatment time		
		3 hours	5 hours	24 hours
96-well	HeLa, HepG2, A549 (1×10^4 cells/well)	10-fold	10-fold	15-fold
	Jurkat (1×10^4 cells/well)		10-fold	10-fold
24-well	HeLa, HepG2, A549 (5×10^4 cells/well)	10-fold	10-fold	20-fold
	Jurkat (1×10^6 cells/well)	10-fold	10-fold	15-fold

Measurement



(1) Add 20 µl of sample solutions to each well.

Note: To obtain accurate data, triplicate measurements are recommended.

(2) Add 80 µl of Lactate working solution to each well.

Note: Because the enzymatic reaction starts immediately after the Lactate working solution is added, use a multichannel pipette to minimize time lags in pipetting.

(3) Incubate the microplate at 37 ° C for 30 minutes.

Note: Seal the microplate during incubation to prevent evaporation.

(4) Measure the absorbance at 450 nm using a microplate reader.

-Preparation of Solutions and Measurement (JC-1 Assay)

Preparation of Imaging Buffer solution

Dilute Imaging Buffer (10×) 10-fold with ddH_2O.

Note: Refer to Table 4 for amounts of Imaging Buffer solution to prepare.

Note: Imaging Buffer solution is used for the washing step and observation.

Note: Please use up the Imaging Buffer solution within that day.

Table 4. Required amount of Imaging Buffer solution by vessel type

	Adherent cells				Suspension cells
Culture equipment	6-well	96-well	ibidi 8 well plate	35-mm dish	1.5-ml microtube
Imaging Buffer solution	2 ml/well	100 µl/well	200 µl/well	2 ml/dish	0.5 ml/tube

Preparation of JC-1 DMSO stock solution (2 mmol/l)

Add 25 µl DMSO to the JC-1 Dye tube and dissolve by pipetting.

Note: The JC-1 DMSO stock solution is stable for 1 month when stored at -20 ° C.

Note: Protect the JC-1 DMSO stock solution from light.

Preparation of JC-1 working solution

(1) Add JC-1 DMSO stock solution to a conical tube.

(2) Add an appropriate amount of medium to the tube prepared in step (1), then mix immediately by pipetting 10 times

Note: Refer to Table 5 for the amounts of solutions to be mixed.

Note: Equilibrate both the JC-1 DMSO stock solution and culture medium to room temperature before preparing the working solution.

Note: To prepare a homogeneous JC-1 working solution, please exactly follow the preparation procedure shown in Figure 2).

Note: Please use up the JC-1 working solution within that day.

Table 5. Examples of JC-1 working solution (per well)

	Adherent cells				Suspension cells
Culture equipment (JC-1 working solution amount)	6-well (1 ml/well)	96-well (50 µl/well)	ibidi 8 well plate (100 µl/well)	35-mm dish (1 ml/dish)	1.5-ml microtube (0.25 ml/tube)
medium	1000 µl	50 µl	100 µl	1000 µl	250 µl
JC-1 DMSO stock solution	6.6 µl	0.33 µl	0.66 µl	6.6 µl	1.6 µl

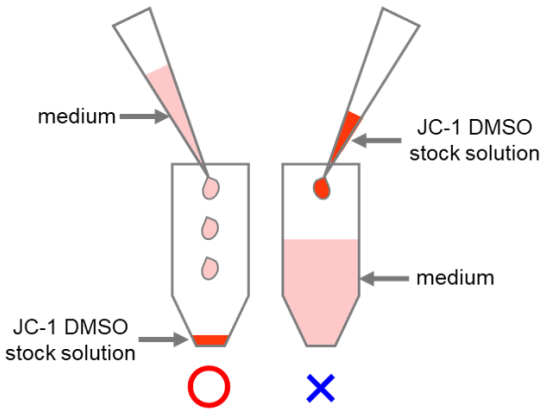


Figure 2. Preparation of JC-1 working solution

Note: Dilute JC-1 DMSO stock solution 150-fold with medium.

Measurement

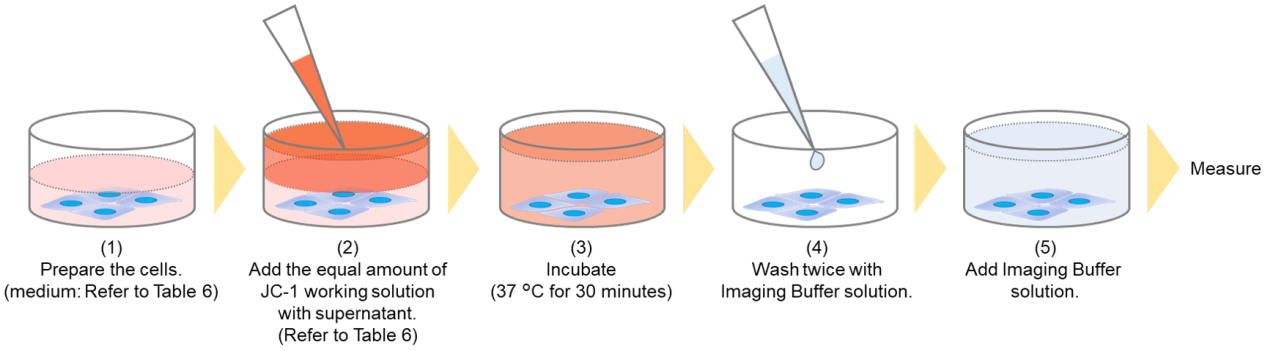


Table 6. The required amount of JC-1 working solution by vessel type

	Adherent cells				Suspension cells
Culture equipment	6-well	96-well	ibidi 8 well plate	35-mm dish	1.5-ml microtube
amount of supernatant (Step (1))	1000 µl	50 µl	100 µl	1000 µl	250 µl
JC-1 working solution (Step (2))	1000 µl	50 µl	100 µl	1000 µl	250 µl

〈For adherent cells〉

(1) Seed cells in a dish or microplate.

(2) Add JC-1 working solution and incubate for 30 minutes in an incubator (37 ° C, 5% CO_2).

(3) Remove the supernatant and wash the cells twice with Imaging Buffer solution.

(4) Add Imaging Buffer solution and observe the cells under a fluorescence microscope or measure the fluorescence intensity using a fluorescence microplate reader.

Note: Do not irradiate the sample with excitation light for a long time. JC-1 dye is light sensitive.

〈For suspension cells〉

(1) Prepare a cell suspension in a 1.5-ml microtube.

(2) Add JC-1 working solution and suspend the cells by pipetting; incubate for 30 minutes in an incubator (37 ° C, 5% CO_2).

(3) Centrifuge at 300 × g for 5 minutes and remove the supernatant.

(4) Add Imaging Buffer solution and resuspend the pellet by pipetting; centrifuge at 300 × g for 5 minutes, and remove the supernatant. Repeat this step twice.

(5) Add Imaging Buffer solution and observe the cells under a fluorescence microscope or measure the fluorescence intensity using a fluorescence microplate reader.

Note: Do not irradiate the sample with excitation light for a long time. JC-1 dye is light sensitive.

-General Protocol

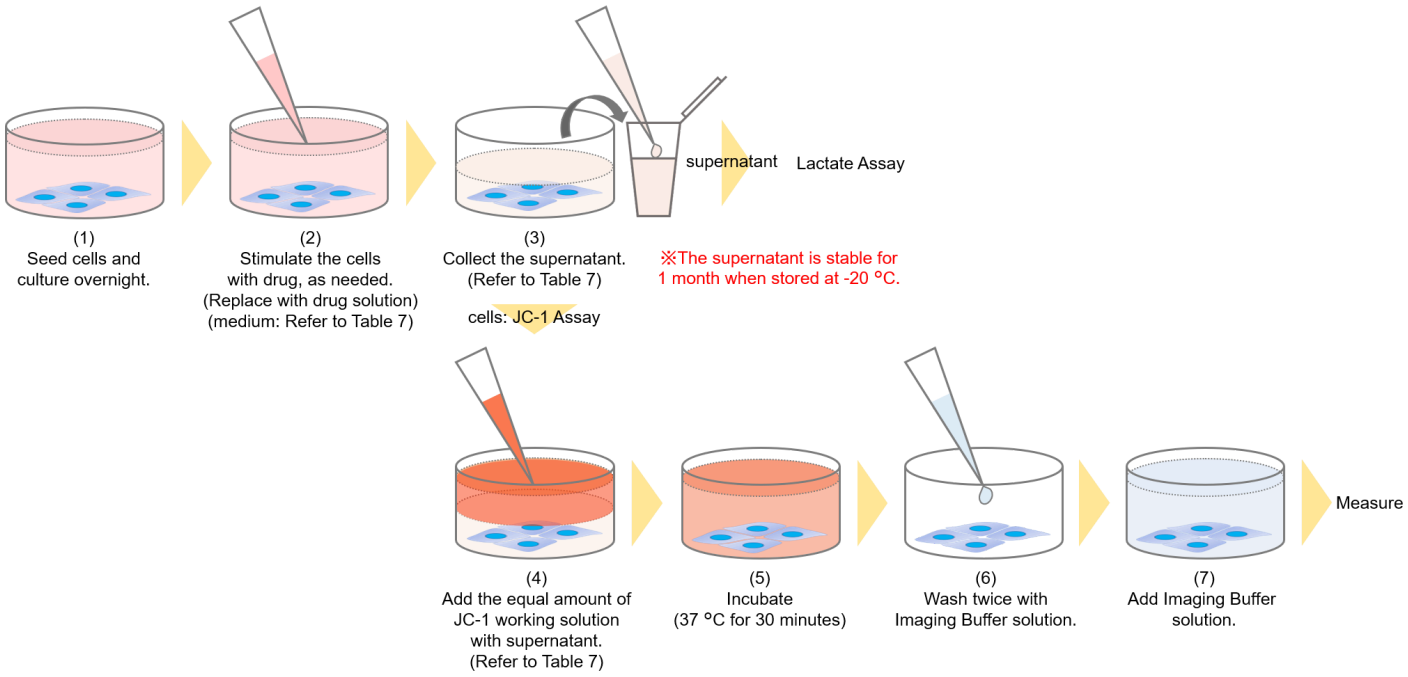


Table 7. Collected amount of supernatant and required amount of JC-1 working solution by vessel type

	Adherent cells				Suspension cells
Culture equipment (Example of cell number)	6-well (2×10^5 cells/well)	96-well (1×10^4 cells/well)	ibidi 8 well plate (2×10^4 cells/well)	35-mm dish (2×10^5 cells/dish)	1.5-ml microtube (1×10^6 cells/tube)
Amount of drug solution (Step (2))	2000 µl	100 µl	200 µl	2000 µl	500 µl
Collected amount of supernatant (Step (3))	1000 µl	50 µl	100 µl	1000 µl	250 µl
JC-1 working solution (Step (4))	1000 µl	50 µl	100 µl	1000 µl	250 µl

〈For adherent cells〉
(1) Seed cells in a dish or microplate, and culture overnight in an incubator (37 ° C, 5% CO_2).
(2) Remove the medium and stimulate the cells with the drug as needed (add drug solution to wells).
(3) Collect half the amount of cell culture supernatant into a 1.5-ml microtube.
(4) Perform the JC-1 Assay using the cells remaining in the dish or microplate.
(5) Perform the Lactate Assay with the supernatant collected in step (3).

〈For suspension cells〉
(1) Prepare a cell suspension in a 1.5-ml microtube.
(2) Centrifuge at 300 × g for 5 minutes and remove the supernatant; stimulate the cells with the drug, as needed (add drug solution to wells).
(3) Centrifuge at 300 × g for 5 minutes.
(4) Collect half the amount of cell culture supernatant into a 1.5-ml microtube.
(5) Perform the JC-1 Assay using the cells remaining in the 1.5-ml microtube.
(6) Perform the Lactate Assay with the supernatant collected in step (4).

-Experimental Example

Lactate production and mitochondrial membrane potential of HeLa cells treated with carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP)

(1) HeLa cells were seeded in a 96-well black microplate (clear bottom, 1×10^4 cells/well in MEM containing 10% fetal bovine serum and 1% penicillin-streptomycin) and cultured overnight in an incubator (37 ° C, 5% CO_2).
(2) The medium was removed, and 0 or 10 µmol/l FCCP solution (100 µl, in MEM containing 10% FBS) was added.
(3) The cells were incubated for 4 hours in an incubator (37 ° C, 5% CO_2).
(4) After incubation, 50 µl of each cell culture supernatant was transferred to a 1.5-ml microtube and diluted 10-fold with ddH_2O to prepare sample

solutions for the Lactate Assay.

Note: For the background control, MEM only was also diluted 10-fold with ddH_2O.

(5) JC-1 working solution (50 µl) was added to the cells remaining in each well of the 96-well black microplate.

(6) The cells were incubated for 30 minutes in an incubator (37 ° C, 5% CO_2).

(7) The medium was removed, and the cells were washed twice with 100 µl of Imaging Buffer solution.

(8) Imaging Buffer solution (100 µl) was added to each well, and the cells were observed under a fluorescence microscope (LSM800, Zeiss Corporation), and the fluorescence intensity was measured using a microplate reader (Infinite M200 PRO, Tecan Trading AG, bottom reading).

(9) The sample solutions (20 µl each) prepared in step (4) were added to individual wells of a 96-well microplate.

(10) Lactate working solution (80 µl) was added to each well.

(11) The assay plate was incubated at 37 ° C for 30 minutes.

(12) The absorbance at 450 nm was measured using a microplate reader, and the absorbance values with and without FCCP treatment were compared.

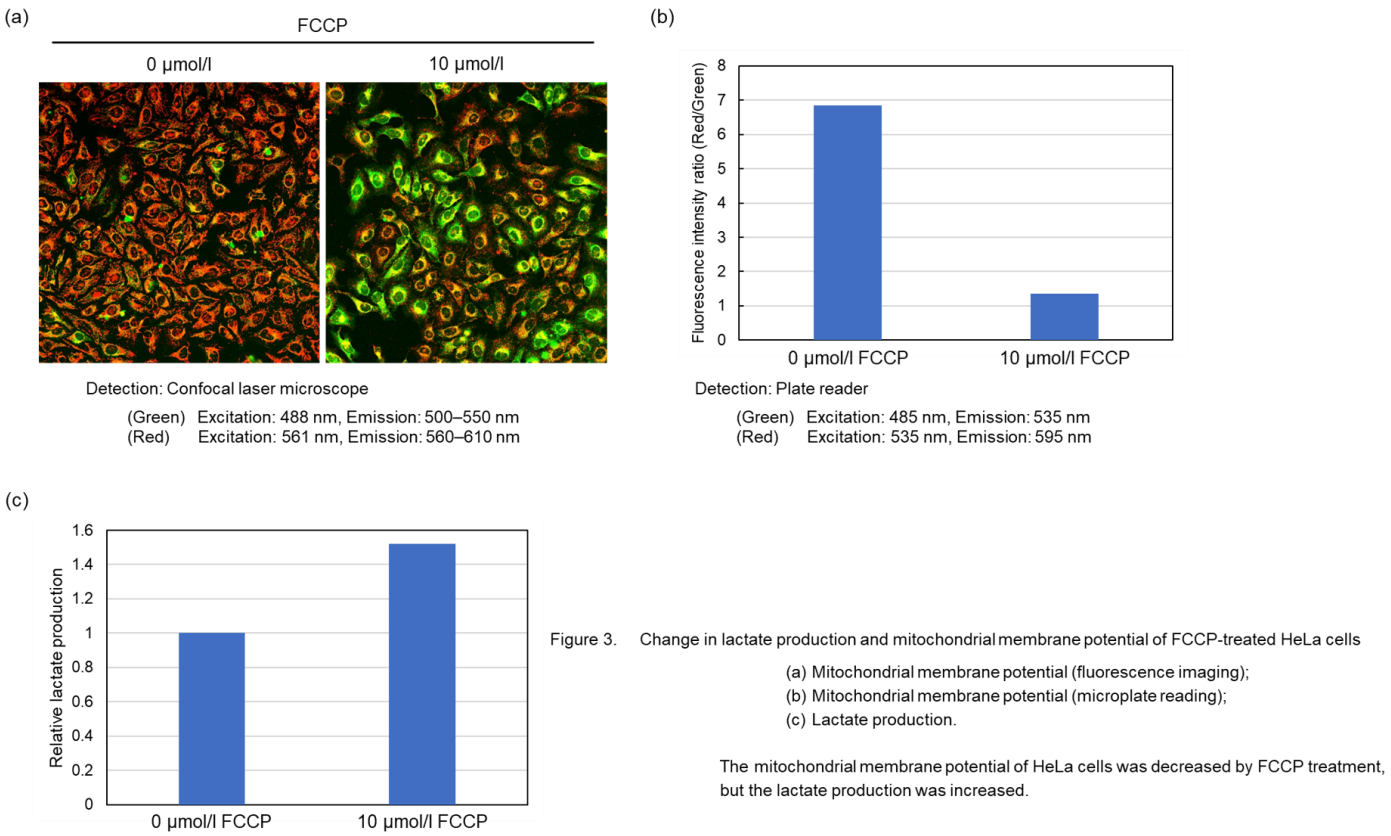


Figure 3. Change in lactate production and mitochondrial membrane potential of FCCP-treated HeLa cells

(a) Mitochondrial membrane potential (fluorescence imaging);
(b) Mitochondrial membrane potential (microplate reading);
(c) Lactate production.

The mitochondrial membrane potential of HeLa cells was decreased by FCCP treatment, but the lactate production was increased.

-Reference

1) E. Liao, et al., Cell Death and Disease, 2014, 5, e1255.
2) R. Shiratori, et al., Sci. Rep., 2019, 9, 18699.

-Frequently Asked Questions / Reference

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Questions

Reference