

Intracellular Oxygen Detection Kit

I306 Product Manual

–General Information

Aerobic organisms mainly produce ATP by mitochondrial oxidative phosphorylation; because oxygen (O_2) is consumed in this process, it is one of the substances that is essential for our energy production. Meanwhile, chronic hypoxia in cells can lead to cancer and ischemic diseases.

The Intracellular Oxygen Detection Kit enables the measurement of oxygen concentration changes (phosphorescence lifetime changes) in cultured cells. The kit includes an Intracellular Oxygen Probe, which increases phosphorescence intensity when the oxygen concentration in the cell decreases, and Mineral Oil to block the influx of oxygen from the air.

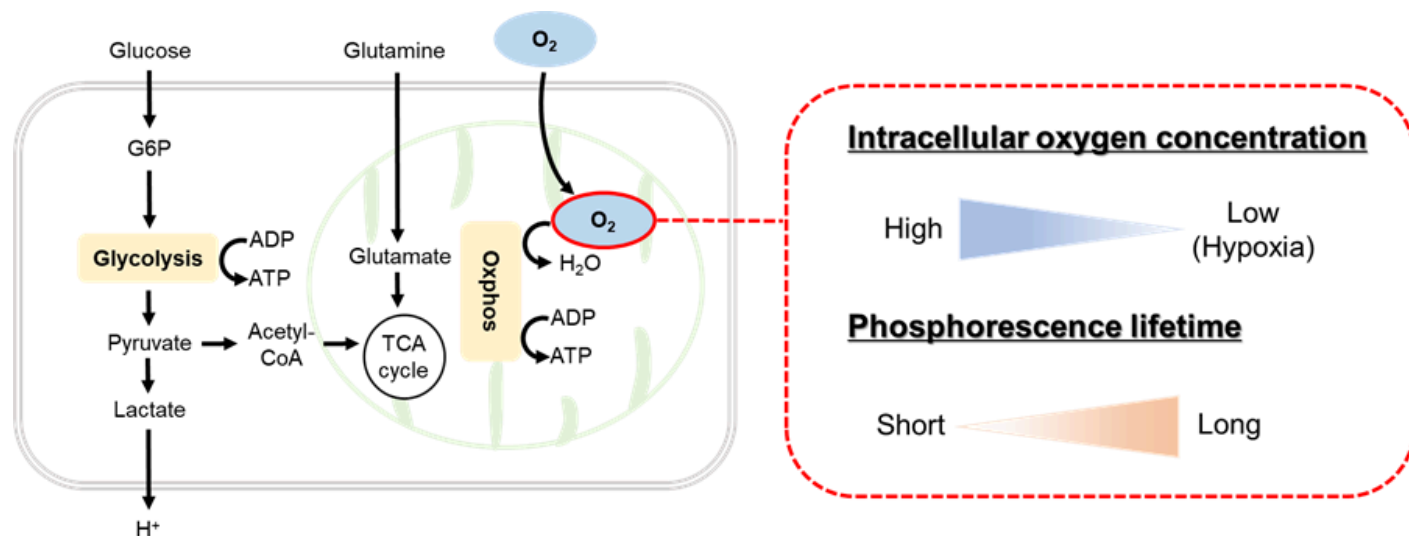


Figure 1. Principle of Intracellular Oxygen Detection Kit

–Kit Contents

	100 tests	300 tests
Intracellular Oxygen Probe	× 1	× 3
Mineral Oil	10 ml × 1	10 ml × 3

–Storage Conditions

Store at $-20^{\circ}C$

–Required Equipment and Materials

- Microplate reader with temperature control and time-resolved fluorescence (TR-F) measurement
- 96-well black clear-bottom microplate
- 20–200 μl multichannel pipette

- 100–1000 μl , 20–200 μl and 2–20 μl micropipettes
- Dimethyl sulfoxide (DMSO)
- 1.5 ml microtube
- Conical tube
- Heating block or water bath

–Precautions

- Equilibrate reagents to room temperature prior to use.
- Briefly centrifuge the Intracellular Oxygen Probe tube before opening to remove all content from the walls of the tube and the inside of the cap.
- Analysis of samples in triplicate is recommended for accuracy.
- Set the microplate reader temperature at 37 ° C before measurement.
- Pre-warm the Mineral Oil and sample solutions to around 37 ° C in a heating block or water bath.
- When using a 96-well microplate, please consider the following.
 Suspension cells (e.g., Jurkat cells): >100,000 cells/well
 Adherent cells (e.g., HepG2 cells): >50,000 cells/well

–Preparation of Solutions

Preparation of Intracellular Oxygen Probe DMSO stock solution

Add 12 μl DMSO to the Intracellular Oxygen Probe tube and dissolve by pipetting.

Note: The Intracellular Oxygen Probe DMSO stock solution is stable for 2 months when stored at –20 ° C and protected from light.

Preparation of a working solution

1. Add medium to a conical tube (see Table 1).
2. Add DMSO stock solution to the medium.

Note: The working solution is light-sensitive and cannot be stored after preparation. Prepare the solution just before use and protect it from light by covering it with aluminum foil.

Table 1. The required amount of working solution by sample number

	24 wells	48 wells	96 wells
DMSO stock solution	3 μl	5.5 μl	11 μl
Medium	2997 μl	5494.5 μl	10989 μl

–General Protocol

〈Suspension cells〉

When testing sample types or concentrations for the same cell line and the number of cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Sample 7								
B	Control											
C	Sample 1											
D	Sample 2											
E	Sample 3											
F	Sample 4											
G	Sample 5											
H	Sample 6			Sample 14								

When testing different cell lines.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Blank								
B	Control			Control								
C	Sample 1			Sample 1								
D												
E												
F												
G												
H							Sample 6			Sample 6		
	Cell line : A			Cell line : B								

When testing different number of cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Blank								
B	Control			Control								
C	Sample 1			Sample 1								
D	↓			↓								
E												
F												
G												
H	Sample 6			Sample 6								
	Number of cells : 1			Number of cells : 2								

Figure 2. Example assay plate formats (n=3)

1. Suspend cells ($>1.0 \times 10^6$ cells/ml) in a working solution in a conical tube.
2. Transfer the cell suspension (1) to a culture vessel and culture in an incubator (37°C , 5% CO_2) for 1 h to stain the cells.
3. Collect the cell suspension (2) in a conical tube, centrifuge, and remove the supernatant.
4. Add medium, suspend sufficiently, centrifuge again, and remove the supernatant.
5. Prepare suspensions of unstained cells in medium ($>1.0 \times 10^6$ cells/ml) as a Blank and stained cells ($>1.0 \times 10^6$ cells/ml) as the Control and Samples (see Table 2).
6. Seed the prepared suspensions (5) (100 μl of each) in a 96-well black clear-bottom microplate ($>1.0 \times 10^5$ cells/well) (see Table 2 and Figure 2).

Note: Triplicate measurements are recommended to obtain accurate data.

Table 2. The required amount of samples and reagents for each well

	Blank	Control	Sample
Unstained cells	100 μl	–	–
Stained cells	–	100 μl	100 μl
Sample solution	–	–	10 μl
Medium	10 μl	10 μl	–
Mineral Oil	1 drop	1 drop	1 drop

7. Incubate the microplate for 10 min in a microplate reader (37°C).

Note: Pre-warm the microplate reader to 37°C .

Note: Incubate in the microplate reader, not an incubator.

8. Measure the intensities before the addition of the sample solution in kinetics mode (10-min interval for 20 min) using the microplate reader set to measure in the following two conditions per well:

1st condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37°C
- Integration Time: 20 μs
- Lag Time: 10 μs

2nd condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 40 µs

9. Take the microplate out of the microplate reader and add 10 µl of medium to Blank and Control wells (see Table 2).

Note: Pre-warm the medium to around 37 ° C in a heating block or water bath.

10. Dilute a sample with medium and add 10 µl to the Sample wells (see Table 2).

Note: Pre-warm the diluted sample solution to around 37 ° C in a heating block or water bath.

11. Immediately after adding the sample solution, add 1 drop of Mineral Oil to all wells.

Note: Pre-warm the Mineral Oil to around 37 ° C in a heating block or water bath.

12. Incubate the microplate for 10 min in a microplate reader (37 ° C).

Note: Pre-warm the microplate reader to 37 ° C.

Note: Incubate in the microplate reader, not an incubator.

13. Measure the intensities after the addition of sample solution in kinetics mode (10-min interval for 200 min) using the microplate reader set to measure in the following two conditions per well:

1st condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 10 µs

2nd condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 40 µs

14. Calculate the phosphorescence lifetime (t_p) using the following equation from the intensity values measured in the two conditions, and plot a graph with horizontal axis time (min) and vertical axis phosphorescence lifetime (µs).

$$\tau_p = \frac{30}{\ln(W1/W2)}$$

$$\left(\begin{array}{l} W1 : \text{Value of intensity measured in the 1}^{\text{st}} \text{ condition} \\ \left. \begin{array}{c} \text{Control} \\ \text{or} \\ \text{Sample} \end{array} \right\} - \text{Blank} \\ \\ W2 : \text{Value of intensity measured in the 2}^{\text{nd}} \text{ condition} \\ \left. \begin{array}{c} \text{Control} \\ \text{or} \\ \text{Sample} \end{array} \right\} - \text{Blank} \end{array} \right)$$

$$= 30/\text{LN}((\text{Control} - \text{Blank})/(\text{Control} - \text{Blank}))$$

or

$$= 30/\text{LN}((\text{Sample} - \text{Blank})/(\text{Sample} - \text{Blank}))$$

<Adherent cells>

When testing sample types or concentrations for the same cell line and the number of cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Sample 7										
B	Control											
C	Sample 1											
D	Sample 2											
E	Sample 3											
F	Sample 4											
G	Sample 5											
H	Sample 6	Sample 14										

When testing different cell lines.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Blank								
B	Control			Control								
C	Sample 1			Sample 1								
D												
E												
F												
G												
H	Sample 6			Sample 6								
	Cell line : A						Cell line : B					

When testing different number of cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Blank								
B	Control			Control								
C	Sample 1			Sample 1								
D												
E												
F												
G												
H	Sample 6			Sample 6								
	Number of cells : 1						Number of cells : 2					

Figure 3. Example assay plate formats (n=3)

1. Seed cell suspension (100 μ l, $>5.0 \times 10^5$ cells/ml) in a 96-well black clear-bottom microplate for the Blank, Control, and Samples.

Note: Triplicate measurements are recommended to obtain accurate data.

2. Culture overnight in an incubator (37 ° C, 5% CO₂).

3. Remove the medium from the Control and Samples and add 100 μ l of working solution.

4. Culture in an incubator (37 ° C, 5% CO₂) for 1 h to stain the cells.

5. Remove the solution (Blank: medium; Control and Samples: working solution) and add 100 μ l of the medium.

6. Remove the medium and add 100 μ l of medium.

7. Incubate the microplate for 10 min in a microplate reader (37 ° C).

Note: Pre-warm the microplate reader to 37 ° C.

Note: Incubate in the microplate reader, not an incubator.

8. Measure the intensities before the addition of sample solution in kinetics mode (10-min interval for 20 min) using the microplate reader set to measure in the following two conditions per well:

1st condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 10 µs

2nd condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 40 µs

9. Take the microplate out of the microplate reader and add 10 µl of medium to Blank and Control wells (see Table 3).

Note: Pre-warm the medium to around 37 ° C in a heating block or water bath.

Table 3. The required amount of samples and reagents for each well

	Blank	Control	Sample
Medium	110 µl	110 µl	100 µl
Sample solution	–	–	10 µl
Mineral Oil	1 drop	1 drop	1 drop

10. Dilute a sample with medium and add 10 µl to the Sample wells (see Table 3).

Note: Pre-warm the diluted sample solution to around 37 ° C in a heating block or water bath.

11. Immediately after adding the sample solution, add 1 drop of Mineral Oil to all wells.

Note: Pre-warm the Mineral Oil to around 37 ° C in a heating block or water bath.

12. Incubate the microplate for 10 min in a microplate reader (37 ° C).

Note: Pre-warm the microplate reader to 37 ° C.

Note: Incubate in the microplate reader, not an incubator.

13. Measure the intensities after the addition of sample solution in kinetics mode (10-min interval for 200 min) using the microplate reader set to measure in the following two conditions per well;

1st condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 10 µs

2nd condition

- Mode: Fluorescence, Bottom reading
- Wavelength: Ex: 480 nm, Em: 590 nm
- Temperature: 37 ° C
- Integration Time: 20 µs
- Lag Time: 40 µs

14. Calculate the phosphorescence lifetime (t_p) using the following equation from the intensity values measured in the two conditions, and plot a graph with horizontal axis time (min) and vertical axis phosphorescence lifetime (µs).

$$\tau_p = \frac{30}{\ln(W1/W2)}$$

$$\left(\begin{array}{l} W1 : \text{Value of intensity measured in the 1}^{\text{st}} \text{ condition} \\ \left. \begin{array}{c} \text{Control} \\ \text{or} \\ \text{Sample} \end{array} \right\} - \text{Blank} \\ \\ W2 : \text{Value of intensity measured in the 2}^{\text{nd}} \text{ condition} \\ \left. \begin{array}{c} \text{Control} \\ \text{or} \\ \text{Sample} \end{array} \right\} - \text{Blank} \end{array} \right)$$

$$= 30/\ln((\text{Control} - \text{Blank})/(\text{Control} - \text{Blank}))$$

or

$$= 30/\ln((\text{Sample} - \text{Blank})/(\text{Sample} - \text{Blank}))$$

-Experimental Example

Changes in intracellular oxygen concentration (measured as phosphorescence lifetime) in HepG2 cells on treatment with the mitochondrial uncoupler FCCP (plate reader detection).

1. HepG2 cells (7.2×10^5 cells/ml) were seeded (100 μ l, 7.2×10^4 cells/well) in a 96-well black clear-bottom microplate.
2. The cells were cultured overnight in an incubator (37 ° C, 5% CO₂).
3. The medium (DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin) was removed from the Control and Sample wells.
4. A working solution (100 μ l/well) was added to the Control and Sample wells of the 96-well black clear-bottom microplate.
5. The microplate was incubated for 1 h in an incubator (37 ° C, 5% CO₂).
6. The solution (Blank: medium; Control and Samples: working solution) was removed from the wells, and the medium (DMEM containing 10% FBS and 1% P/S) was added (100 μ l/well).
7. The medium was removed from the wells, and the medium (DMEM containing 10% FBS and 1% P/S) was added (100 μ l/well).
8. The microplate was incubated at 37 ° C for 10 min in the microplate reader.
9. Kinetics were measured using the microplate reader (Ex: 480 nm, Em: 590 nm, 10-min interval for 20 min, Bottom reading, two condotions).
10. The following additions were made: Blank and Control wells: medium (DMEM containing 10% FBS and 1% P/S) (10 μ l/well); Sample 1: FCCP diluted in medium (DMEM containing 10% FBS and 1% P/S) (10 μ l/well); Sample 2: Antimycin diluted in medium (DMEM containing 10% FBS and 1% P/S) (10 μ l/well).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	Control											
C	Sample 1 (FCCP)											
D	Sample 2 (Antimycin)											
E												
F												
G												
H												

Figure 4. Experimental example assay plate formats (n=3)

11. Mineral Oil (1 drop) was added to all wells.
12. The microplate was incubated at 37 ° C for 10 min in the microplate reader.
13. Kinetics were measured using the microplate reader (Ex: 480 nm, Em: 590 nm, 10-min interval for 200 min, Bottom reading, two conditions).
14. Phosphorescence lifetime was calculated from the intensity measurements.

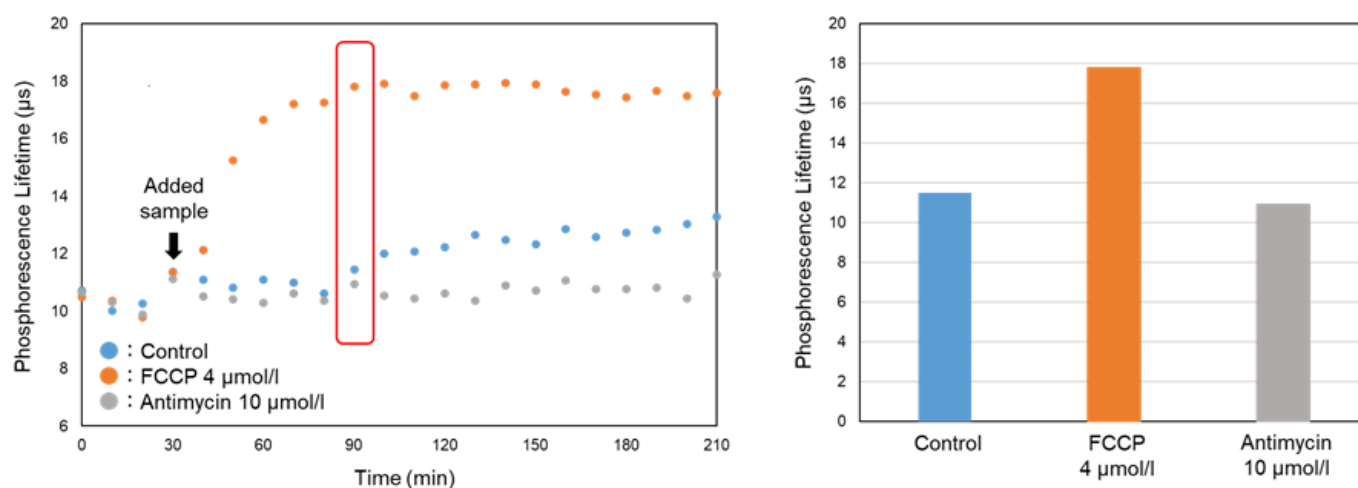
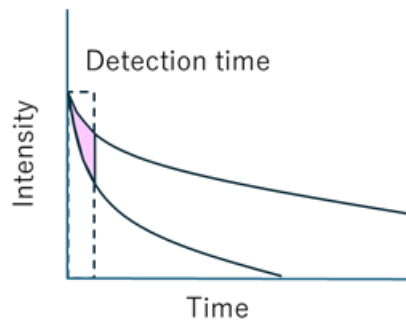


Figure 5. The changes in phosphorescence lifetime treated FCCP (uncoupler)

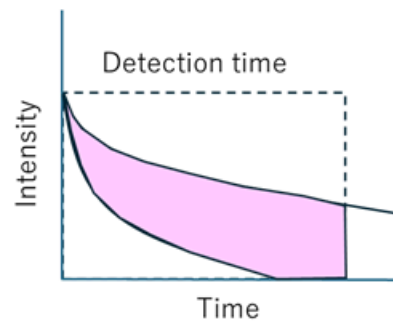
The phosphorescence lifetime was increased by FCCP treatment compared with control cells.

Changes in intracellular oxygen concentration in HepG2 cells on treatment with the mitochondrial uncoupler FCCP (imaging detection).

Note: Since phosphorescence has a longer emission time than fluorescence, the phosphorescence signal cannot be adequately detected if the detection time per pixel is too short. The detection time per pixel must be considered to detect the oxygen responsiveness of phosphorescence using confocal fluorescence microscopy (see Figure 6). Since the optimum detection time is different for each device and detection condition, please perform a preliminary experiment to evaluate the detection time per pixel before this experiment. The following experiments are performed using a ZEISS confocal fluorescence microscope (model: LSM 800).



Small difference in phosphorescence intensity between condition A (normal oxygen level) and condition B (hypoxia).



Large difference in phosphorescence intensity between condition A (normal oxygen level) and condition B (hypoxia).

Figure 6. Effect of detection time on oxygen detection using phosphorescence

– Preliminary Experiment (optimization of scan speed) –

1. HepG2 cells (6.0×10^5 cells/ml) were seeded ($100 \mu\text{l}$, 6.0×10^4 cells/well) in a 3-well glass-base dish (IWAKI, code: 3970-103).

Note: Using an imaging vessel with well that are round in shape and about the same size as a 96-well plate (see Figure 7). μ -slide 8 well plate (ibidi) with square wells or a 5 cm glass-base dish will not allow the oil to penetrate all the way to the wells.

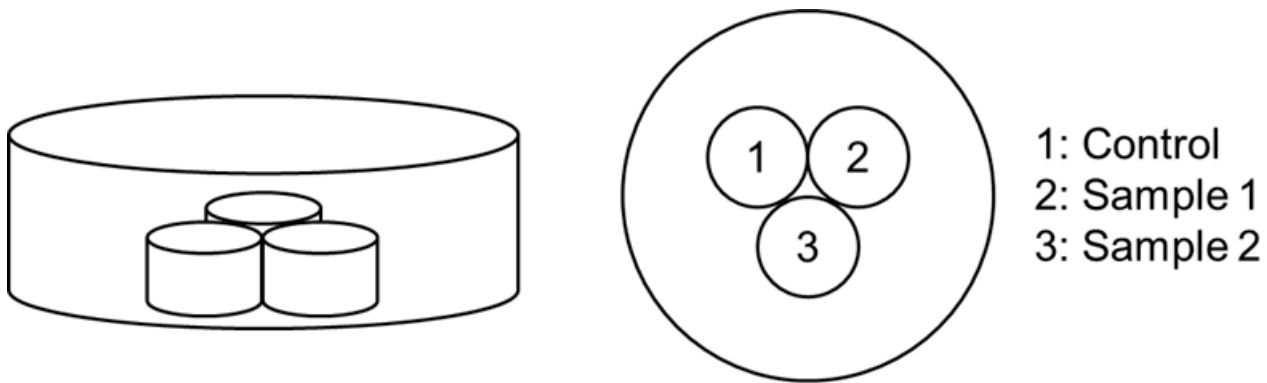


Figure 7. The imaging vessel

2. The medium (DMEM containing 10% fetal bovine serum and 1% penicillin–streptomycin) (2 ml) was added to the outside of the wells to prevent drying.
3. The cells were cultured overnight in an incubator (37°C , 5% CO_2).
4. The medium was removed from the Control and Sample wells.
5. A working solution ($100 \mu\text{l}$ /well) was added to the Control and Sample wells of the 3-well glass-base dish.
6. The dish was incubated for 1 h in an incubator (37°C , 5% CO_2).
7. The solution (Control and Sample: working solution) was removed from the wells, and a medium (DMEM containing 10% FBS and 1% P/S) was added ($100 \mu\text{l}$ /well).
8. The medium was removed from the wells, and the medium (DMEM containing 10% FBS and 1% P/S) was added ($100 \mu\text{l}$ /well).
9. The dish was incubated at 37°C for 10 min in the incubator.
10. The following additions were made: Control: medium (DMEM containing 10% FBS and 1% P/S) ($10 \mu\text{l}$ /well); Sample 1: FCCP diluted in medium (DMEM containing 10% FBS and 1% P/S) ($10 \mu\text{l}$ /well) (see Figure 7).
11. Mineral Oil (1 drop) was added to all wells.
12. The dish was incubated at 37°C for 120 min in the incubator.
13. The cells were observed using the confocal fluorescence microscope under multiple scan speed conditions.

Scan Speed	9	3
Pixel Dwell	0.52 μ s	16.48 μ s
Scan Time	5.06 sec	1 min 46 sec

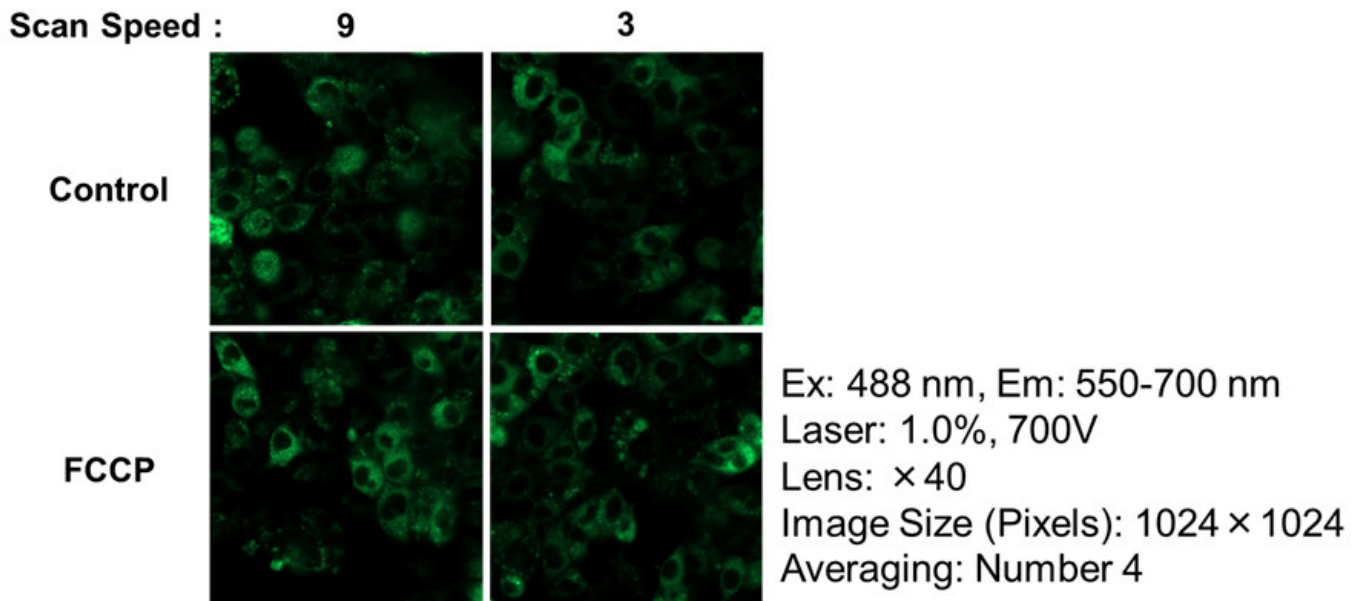


Figure 8. The changes of phosphorescence intensity treated FCCP under multiple scan speed conditions

The preliminary experiment result confirmed the difference between Control and FCCP under Scan Speed: 3 rather than under Scan Speed: 9.

So, this experiment confirmed the time-dependent intensity change by FCCP under Scan Speed: 3.

– Experiment (time-dependent intensity change by FCCP) –

1. HepG2 cells (6.0×10^5 cells/ml) were diluted in medium (DMEM containing 10% FBS and 1% P/S) and seeded (100 μ l, 6.0×10^4 cells/well) in a 3-well glass-base dish (IWAKI, code: 3970-103).
2. The medium (DMEM containing 10% FBS and 1% P/S) (2 ml) was added to the outside of the wells to prevent drying.
3. The cells were cultured overnight in an incubator (37 ° C, 5% CO₂).
4. The medium was removed from the Control and Sample wells.
5. A working solution (100 μ l/well) was added to the Control and Sample wells of the 3-well glass-base dish.
6. The dish was incubated for 1 h in an incubator (37 ° C, 5% CO₂).
7. The solution (Control and Sample: working solution) was removed from the wells, and a medium (DMEM containing 10% FBS and 1% P/S) was added (100 μ l/well).
8. The medium was removed from the wells, and the medium (DMEM containing 10% FBS and 1% P/S) was added (100 μ l/well).
9. The dish was incubated at 37 ° C for 10 min in the incubator.
10. The following additions were made: Control: medium (DMEM containing 10% FBS and 1% P/S) (10 μ l/well); Sample 1: FCCP diluted in medium (DMEM containing 10% FBS and 1% P/S) (10 μ l/well) (see Figure 7).
11. Mineral Oil (1 drop) was added to all wells.
12. The cells were observed using the confocal fluorescence microscope under scan speed: 3 conditions.

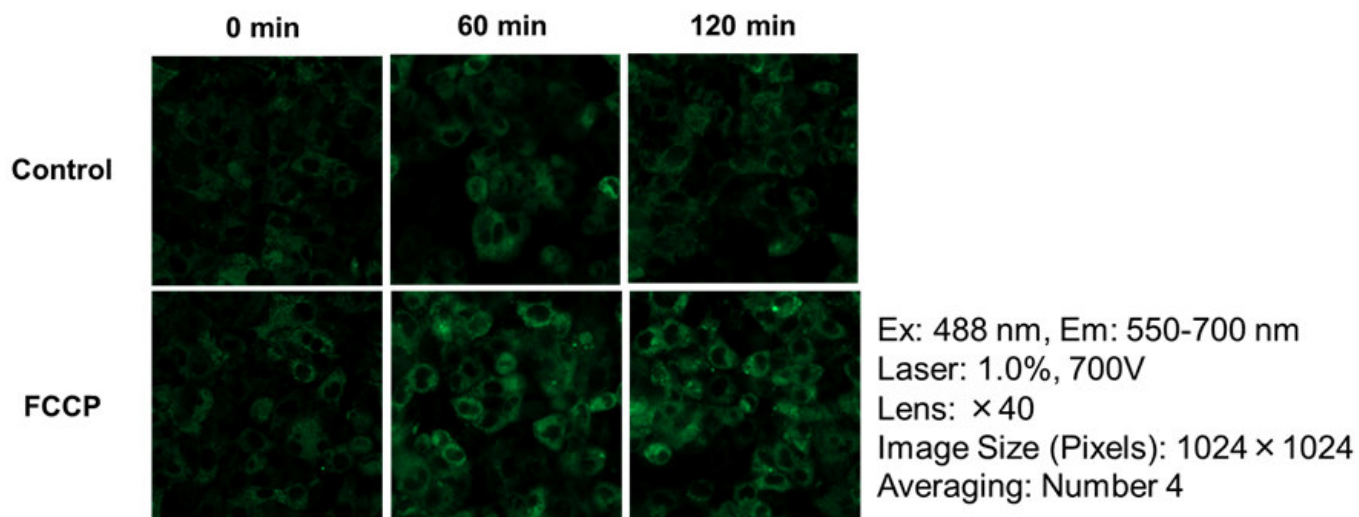


Figure 9. The changes of phosphorescence intensity treated FCCP under Scan Speed: 3
 The changes in the intensity of Control and FCCP were confirmed at 60 min and 120 min after FCCP stimulation.