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Differential dependence on oxygen tension during the maturation process between monomeric Kusabira Orange 2 and monomeric Azami Green expressed in HeLa cells

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ABSTRACT

Although oxygen is required for functional chromophore formation during the maturation process of fluorescent proteins, the effects of hypoxia on their fluorescence have rarely been studied in mammalian cells. We recently reported that severe hypoxia ($pO_2 < 0.1\%$) abrogates fluorescence from the fluorescent ubiquitination-based cell cycle indicator (Fucci) expressed in HeLa cells. Fucci is a system for visualizing cell cycle progression in live cells using red (monomeric Kusabira Orange 2, mKO_2) and green (monomeric Azami Green, mAG) fluorescent proteins. In this study, taking advantage of the system, we attempted to determine the dependence on oxygen tension (pO_2) of these two fluorescent proteins during the maturation process. The oxygen tension at which the number of fluorescence-positive cells was reduced by 50% (pO_2·50) was 0.9% and 0.3% for mKO2 and mAG, respectively. Furthermore, we measured fluorescence recovery kinetics after reoxygenation in cells treated at two different pO_2 levels, and observed that mKO2 exhibits slower kinetics of oxidation than mAG. Thus, we demonstrate that mKO2 exhibits a stronger dependence on oxygen tension than mAG, as well as the usefulness of this novel method to produce varying levels of hypoxic conditions.

1. Introduction

Fluorescent proteins are widely used to visualize cellular events such as subcellular localization and gene expression. Each fluorescent protein contains an intrinsic chromophore, which must mature in order to emit fluorescence. Chromophore maturation consists of three major steps: protein folding, cyclization of the tripeptide chromophore motif, and oxidation of the cyclized chromophore [1,2]; molecular oxygen is essential for the oxidation that occurs in the third step [3–5]. However, the effect of hypoxia on the generation of fluorescence has rarely been studied in mammalian cells; to the best of our knowledge, there exists only one report, by Vordemark et al. [6]. That study demonstrated that the fluorescence intensity of EGFP under control of the hypoxia responsive element in a human fibrosarcoma cell line (HT1080) decreased under severe hypoxic conditions.

The fluorescent ubiquitination-based cell cycle indicator (Fucci) was developed to visualize cell cycle dynamics in live cells [7]. This system comprises two fusion proteins: monomeric Kusabira Orange 2 (mKO2) fused to amino acids 30–120 of Cdt1, which contain a binding domain for E3 ubiquitin ligase, SCF^{Skp2}; and monomeric Azami Green (mAG), fused to amino acids 1–110 of Geminin, which contain a binding domain for E3 ubiquitin ligase, APC/C^{Cdh1}. We recently reported that severe hypoxia ($pO_2 < 0.1\%$) almost completely abrogates the fluorescence of both proteins in HeLa cells expressing the Fucci probes (HeLa-Fucci) after 10–16 h, despite the fact that expression levels of both fluorescent proteins are maintained [8]. However, fluorescence was not affected in HeLa cells expressing only fluorescent proteins without the E3 ligase-binding regions. We concluded that this phenomenon was caused by a combination of degradation of already-synthesized and oxidized proteins, according to the design of the Fucci system, and the generation of newly synthesized, non-oxidized proteins following hypoxic treatment. This experimental model is thus suitable to test how hypoxia influences the generation of mature fluorescent proteins. In this study, taking advantage of the Fucci system, we explored the detailed kinetics of the oxidation process of mKO2 and mAG. For this purpose, we generated varying levels of oxygen tension by employing a novel simple method that has recently become commercially available.

2. Materials and methods

2.1. Cell line and culture condition

HeLa cells expressing the Fucci probes (HeLa-Fucci) were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Cells were maintained in DMEM (Sigma–Aldrich, St. Louis, MO) with 100 units/ml penicillin and 100 μg/ml strepto-

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mycin supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Hypoxic treatments

To establish hypoxic conditions at varying oxygen tensions, we applied the BIONIX-1 hypoxic culture kit (Sugiyamagen, Tokyo, Japan). This system comprises an AnaeroPack-Anaero 5% (oxygen absorber; Mitsubishi Gas Chemical, Tokyo, Japan), an OXY-1 oxygen monitor (JIKCO, Tokyo, Japan), an AnaeroPouch (Mitsubishi Gas Chemical), and plastic clips for sealing the pouch. Cells were treated according to the manufacturer's instructions, except that the oxygen monitor was pre-treated with an AnaeroPouch-Keep (Mitsubishi Gas Chemical) overnight before the experiment in order to reduce the pO_2 of the air present inside the oxygen monitor. Briefly, the oxygen absorber, cells grown in the plastic dish, and the oxygen monitor were arranged in the pouch as shown in Fig. 1A, and the left open side was sealed with a clip (Fig. 1A, 0 h). The pO_2 in the pouch rapidly decreased after sealing; at the desired pO_2, the pouch was further sealed between the culture dish and the oxygen absorber, using another clip to stop the oxygen absorption (Fig. 1A, Separation). Just before separation by the clip, the air in the culture dish was replaced with the air in the pouch by opening the lid of the dish in order to accelerate the equilibration. After separation, the pouch was maintained at 37 °C for 16 h. For a control at atmospheric pO_2, cells were sealed and maintained in an identical apparatus lacking the oxygen absorber. Cells maintained under the most severe hypoxic conditions, obtained by keeping the absorber for 16 h without separation (pO_2 < 0.1%, below the detection sensitivity of the monitor), were also studied.

2.3. Fluorescent imaging

Fluorescent images were taken using a BIOREVO BZ-9000 fluorescence microscope (KEYENCE, Osaka, Japan) immediately after each hypoxic treatment. For quantitative analysis of fluorescent cells, the percentages of red, green, orange (both red and green), and non-fluorescent cells were determined by counting at least 200 cells. Fluorescence-positive cells were defined as those showing recognizable fluorescence, even if the intensity was quite low. Conversely, non-fluorescent cells were defined as those showing almost complete loss of fluorescence under conditions appropriate for observation under the fluorescence microscope. The percentages of fluorescence-positive or -negative cells were plotted against pO_2. The curves for fluorescence-positive cells were further re-plotted by normalizing the percentages of fluorescence-positive cells in atmosphere to 100%. The sigmoid curves were fitted to the following equation (the Gompertz function) using KaleidaGraph 3.6 (Synergy Software, Reading, PA):

$$Y = K \cdot a^{exp(-bX)}$$

where Y represents the percentage of fluorescent cells, X represents pO_2, and K, a and b represent fitting parameters. The pO_2·50, defined as the pO_2 at which the percentage of fluorescence-positive cells was reduced to 50%, was determined for each fluorescent protein.

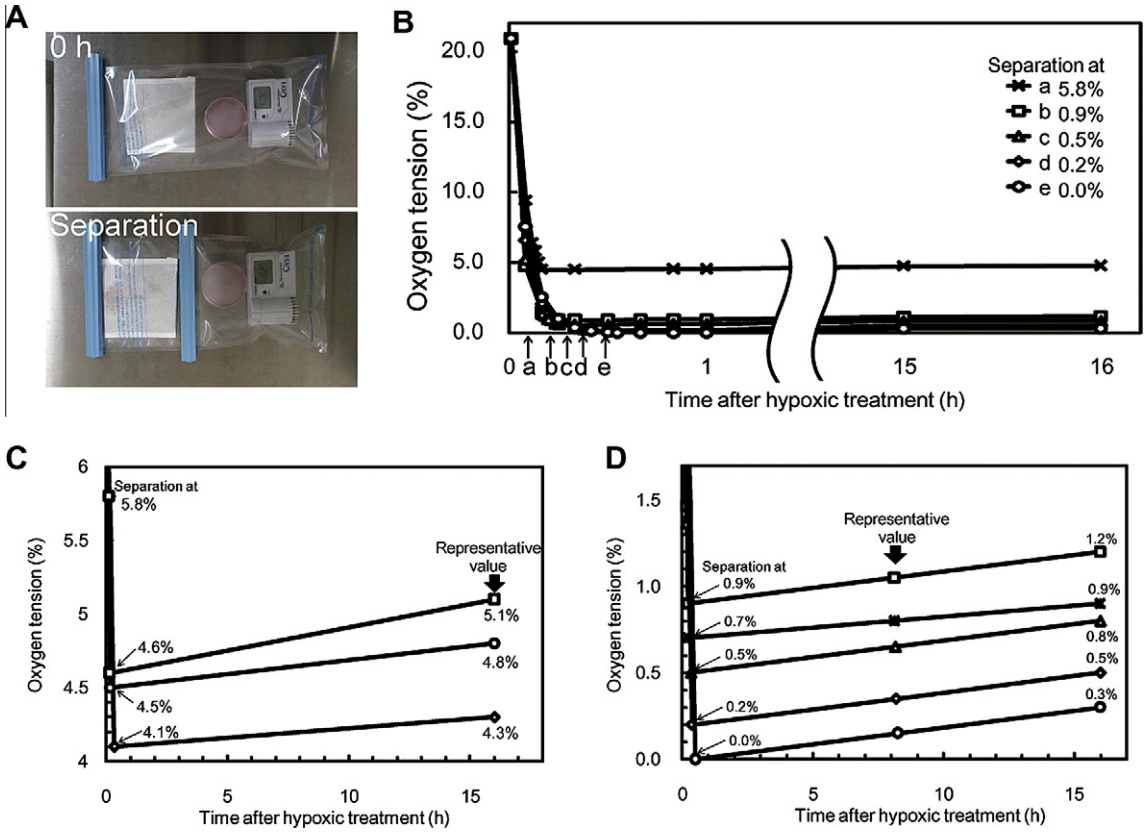

Fig. 1. A simple novel method for producing varying levels of hypoxia, and kinetics of pO_2 reduction. A, Procedure for producing varying levels of hypoxia. 0 h: Sealing the pouch containing an oxygen absorber, a culture dish, and an oxygen monitor with a clip. Separation: Separating between the culture dish and the oxygen absorber using another clip at a specified level of pO_2, in order to stop oxygen absorption. B, Time courses of pO_2 in the pouch during various hypoxic treatments. Arrows indicate the time of separation at varying oxygen tensions (a–e). C, Time courses of pO_2 from the time of separation to the end of hypoxic treatment at pO_2 levels of ~5%. The representative pO_2 values were defined as the pO_2 values 16 h after the separation. D, Time courses of pO_2 from the time of separation to the end of hypoxic treatment at pO_2 levels of <~1%. The representative pO_2 values were defined as the mean of the pO_2 values at separation and those 16 h after separation.

2.4. Quantitative analysis of fluorescence recovery after reoxygenation

Immediately after hypoxic treatment for 16 h, cells were treated with cycloheximide (CHX, 20 μg/ml) and visualized using a microscope equipped with a time-lapse imaging system. For time-lapse imaging, cells were held in an incubation chamber at 37 °C in a humidified atmosphere containing 5% CO_2 (Tokai Hit, Fujinomiya, Japan). For quantitative analysis of fluorescence recovery, the percentages of fluorescent cells were determined as described above, except that at least 100 cells were counted, and the percentages were adjusted by normalizing the maximum percentage of fluorescence-positive cells to 100%. The normalized percentages were plotted against time after reoxygenation.

2.5. Flow cytometric analysis

Immediately after hypoxic treatments, culture medium and trypsinized cells were collected together and centrifuged. Cells were suspended in pre-warmed DMEM containing 2% FBS and 10 mM HEPES (pH 7.5), and incubated in 10 μg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA) for 30 min at 37 °C. After staining, cells were washed twice in PBS containing 5% FBS. Finally, single-cell suspensions were passed through a nylon mesh. Each sample was analyzed using a FACSCanto™II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell cycle distribution was analyzed using the FlowJo software (Tree Star, Ashland, OR).

2.6. Statistical analysis

Mean values were statistically compared using the two-tailed t-test. p values <0.05 were considered statistically significant.

3. Results

3.1. Establishment of hypoxic conditions at varying pO_2 levels

Time courses of pO_2 levels in the pouch after the hypoxic treatment, along with the timing of separation, are shown in Fig. 1B. We noticed that the time courses exhibited different patterns depending on the pO_2 levels at the separation. At the pO_2 levels of 3–5%, the values further decreased after separation for a few minutes and reached a nadir. The levels then slowly increased during incubation for 16 h and reached values about 0.3% higher than at the nadir (Fig. 1C). At pO_2 levels <~1%, the values did not drop, but slowly increased and generally reached levels ~0.3% higher than those at separation (Fig. 1D). Although we have to know the properties of the pO_2 change after the separation depending on the required pO_2 levels, we could reproducibly control varying levels of hypoxic conditions within a small range using the BIONIX-1 system.

Considering the kinetics, representative pO_2 values for each treatment level were defined as follows: (1) for pO_2 levels of 3–5% at separation, the final pO_2 values were recorded 16 h after the separation; (2) for pO_2 levels <1% at separation, the final pO_2values were taken as the mean of the values at separation and the values 16 h after separation.

3.2. Principles of the system to determine dependence on oxygen tension

The characteristics of fluorescence emission of the Fucci system under severe hypoxic conditions (pO_2 < 0.1%) are shown in Fig. 2A [8]. Mature mKO2 and mAG proteins synthesized prior to hypoxic treatment are degraded at the beginning of S phase and at the end of M phase, respectively; non-fluorescent and immature proteins are generated during the hypoxic treatment. Thus, the percentages of fluorescence-positive cells gradually decreased and reached almost zero after roughly half of a cell cycle time following the start of hypoxic treatment (cell cycle time of HeLa-Fucci cells = ~20 h; percentage of cells in G1 = ~50%)[8]. After clearance of the mature fluorescent proteins, according to the design of the Fucci system, we were able to visualize the effect of the degree of hypoxia on the generation of mature proteins. Conversely, the oxidation kinetics after reoxygenation of the immature proteins generated under hypoxic conditions also provide important information regarding oxidation velocity; in our previous study, green fluorescence appeared faster than red fluorescence (Fig. 2B) [8]. This system thus allows us to identify the details of the dependence on oxygen tension of the maturation of mKO2 and mAG when cells are treated under varying pO_2 levels.

The model system described above works only when the cell cycle is not perturbed. We therefore examined the effect of varying levels of hypoxia on cell cycle kinetics. We confirmed that cell cycle distribution was not significantly affected under the experimental conditions used in our study (Fig. 2C).

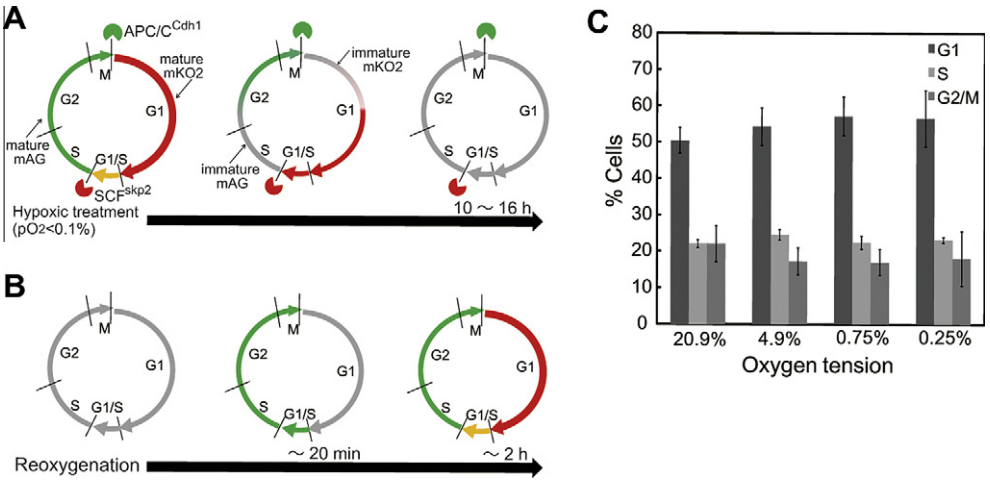

Fig. 2. Principles of the system for analyzing the dependence of mKO_2 and mAG on oxygen tension. A, Schematic presentation of the mechanism of fluorescence loss in the Fucci system during severe hypoxia. B, Schematic presentation of maturation of immature fluorescent proteins generated by severe hypoxia, following reoxygenation. C, Cell cycle distribution under varying levels of pO_2. Cells were treated under varying levels of pO_2 for 16 h and prepared for flow cytometric analysis. Data presented are the mean ± SD of three independent experiments.

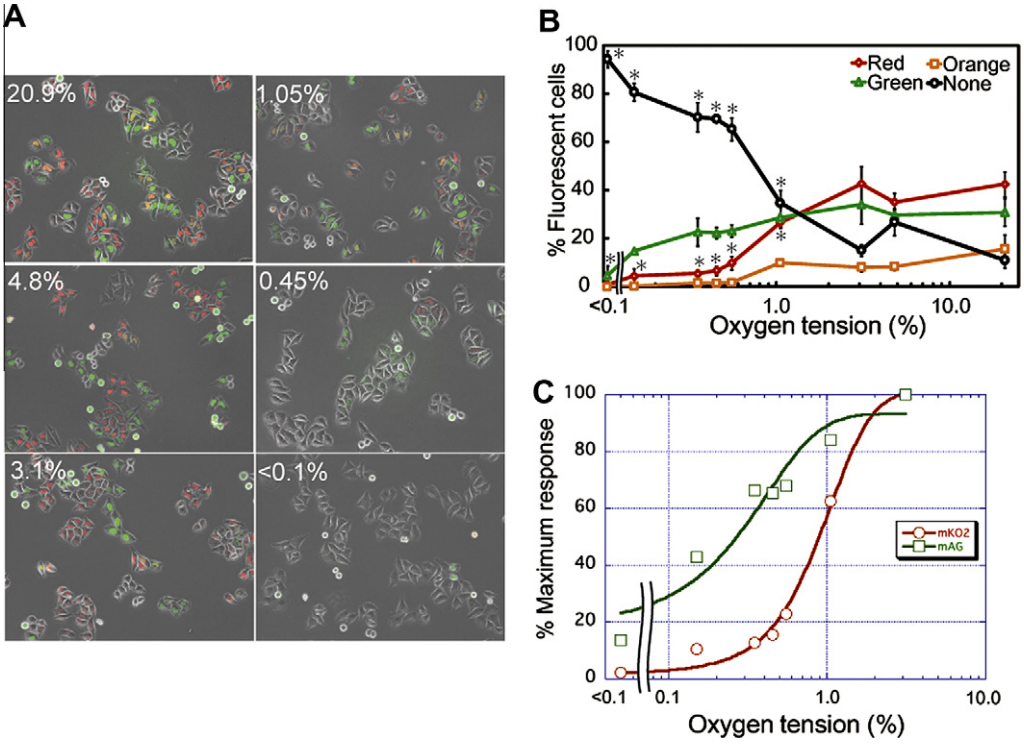

Fig. 3. Kinetics of fluorescence loss at varying pO_2 levels. A, Representative images of fluorescence at varying pO_2 levels. The values indicate the representative pO_2 in each treatment. Cells were treated for 16 h under varying levels of pO_2. B, Percentages of red, green, orange (both red and green), and non-fluorescent cells at various pO_2 levels. The values at $pO_2 < 0.1\%$ are also included. Data presented are the mean ± SD of three independent experiments. $^{*}p < 0.01$ vs. 20.9%. Red and None: 1.2%, 0.55%, 0.45%, 0.35%, 0.15%, and < 0.1%. Green:<0.1%; $p < 0.05$ vs. 20.9%. Orange: 0.45%, 0.35%, 0.15%, and < 0.1%. Green: 0.15%. None: 4.8%. Symbols representing $p < 0.05$ are omitted from the figure for clarity. C, Normalized sigmoid curves fitted to the equation $Y = K \cdot a^{\exp(-bX)}$ as the Gompertz function. The original curves for fluorescence-positive cells were normalized by adjusting the percentages in atmosphere to 100%. The values at $pO_2 < 0.1\%$ were plotted as $pO_2 = 0.05\%$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

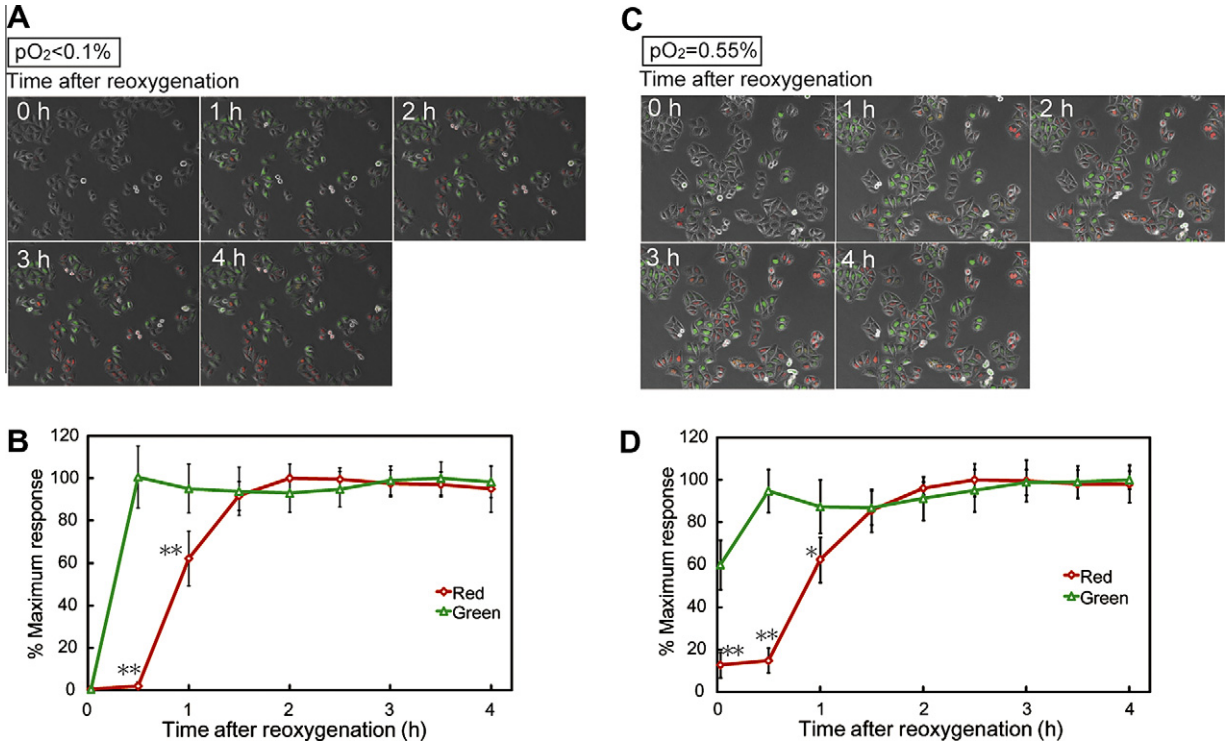

Fig. 4. Fluorescence recovery kinetics after reoxygenation of cells exposed to hypoxia at different pO_2 levels. A, Time-lapse imaging of fluorescence recovery after reoxygenation of cells exposed to hypoxia at $pO_2 < 0.1\%$. Cells were reoxygenated in the presence of 20 μg/ml CHX. B, Percent maximum responses of red and green fluorescence-positive cells after reoxygenation, as shown in Fig. 4A. C, Time-lapse imaging of fluorescence recovery after reoxygenation of cells exposed to hypoxia at $pO_2 = 0.55\%$. Cells treated at $pO_2 = 0.55\%$ for 16 h were reoxygenated in the presence of 20 μg/ml CHX. D, Percent maximum responses of red and green fluorescence-positive cells after reoxygenation, as shown in Fig. 4C. Data presented are the mean ± SD of four independent fields. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus green at the same time after reoxygenation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Kinetics of fluorescence loss at varying levels of pO_2

We next applied the novel method described above to the present study. Cells were treated under varying levels of hypoxia for 16 h, and the fluorescence of each protein was observed immediately after treatment (Fig. 3A and B). Under hypoxic conditions at $pO_2 \geqslant 3\%$, the percentages of cells emitting each fluorescence were not significantly affected. However, when pO_2 levels decreased to <3%, the percentages of cells fluorescing red or green gradually decreased, with a steeper slope for red than green. Neither form of fluorescence was observed under hypoxia at $pO_2 < 0.1\%$, consistent with our previous report (Fig. 3A) [8]. The pO_2 values reducing the percentage of fluorescent cells to 50%, designated $pO_2{\cdot}50$, were 0.9% and 0.3% for mKO2 and mAG, respectively (Fig. 3C).

3.4. Recovery kinetics of fluorescence upon reoxygenation of non-fluorescent cells treated with hypoxia at different levels of pO_2

We next performed reoxygenation experiments in the presence of CHX, using cells subjected to 16-h hypoxic treatment at $pO_2 < 0.1\%$ or $pO_2 = 0.55\%$. In the latter case, only red fluorescence was severely affected, albeit with a low density of green fluorescence (Figs. 3 and 4). When cells were reoxygenated after hypoxic treatment at $pO_2 < 0.1\%$, the percentages of cells fluorescing red or green increased, with a faster rate for green, reaching the similar levels as for cells in an atmosphere (Fig. 4B). The percentages of green-positive cells reached maximum levels within 30 min, but red-positive cells took ~2 h. Following treatment at $pO_2 = 0.55\%$, percentages of red-positive cells recovered slowly and again took ~2 h to reach the maximum levels (Fig. 4D). Green-positive cells quickly recovered from 60% to maximum levels within 30 min. Most of the non-fluorescent cells produced at this pO_2 level were thus determined to be G1 cells expressing non-oxidized mKO2. We confirmed the slower oxidation kinetics of mKO2 relative to mAG, using a different endpoint and different pO_2 levels than in our previous report [8].

4. Discussion

Our experimental model using the Fucci system (Fig. 2) allowed us to test the dependence on oxygen tension during the maturation process of mKO2 and mAG. We determined that $pO_2{\cdot}50$ values were 0.9% and 0.3% for mKO2 and mAG, respectively, and that the recovery kinetics of fluorescence after reoxygenation were much slower for mKO2 than mAG. Taken together with our previous results at $pO_2 < 0.1\%$, these findings provide compelling evidence that maturation of mKO2 is much more strongly dependent on oxygen tension than that of mAG.

The crystal structures of mKO2 and mAG, including their chromophores, have been analyzed in detail [9,10]. The completion of the maturation steps of mKO2 results in formation of a novel three-ring chromophore, consisting of the phenolate ring, the imidazolinone, and the 3-thiazoline ring. It should be noted that these maturation steps, including oxidation, are considerably complicated because two distinct oxidation processes are involved [9]. Further study is needed to determine whether the two oxidation processes truly explain the slow oxidation of mKO2 that we observed in this study.

In general, hypoxic conditions are produced by exchanging atmospheric air with a gas mixture of 95% N_2/5% CO_2 within a specially equipped chamber; it is usually difficult to precisely control the hypoxic levels at arbitrary values of pO_2 [11,12]. In this study, we employed a simple novel method, BIONIX-1. Using this method, varying levels of hypoxic conditions could be easily generated without the use of large or expensive equipment, although the question of small pO_2 fluctuations following separation still remains to be addressed. We believe that this simple method will also be quite useful in other fields including radiobiology, in applications where radiation exposure must be performed under hypoxic conditions.

Fucci has the potential to be quite useful in many fields, including cancer biology. Our group and others have measured fluorescence kinetics using the Fucci system following treatments with specific anti-tumor agents both *in vitro* and *in vivo* [13–15]. Especially in solid tumors, hypoxic fractions occur; these are known to play a key role in causing therapeutic resistance or tumor progression [16]. Although the properties of Fucci in hypoxic fractions of solid tumors are not yet well characterized, the findings reported here suggest that behavior of Fucci could indeed be affected, and therefore must be carefully interpreted, given the differential dependence on pO_2 between mKO2 and mAG.

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