Functional validation of a modified FUCCI cell cycle sensor in a pancreatic cancer cell line

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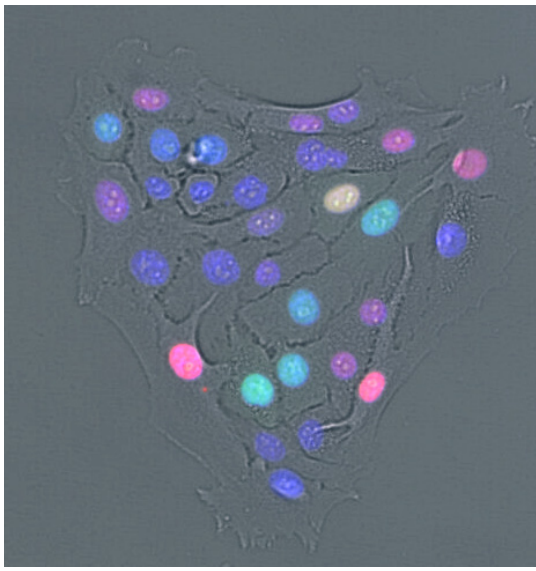
ABSTRACT



In many diseases, the cell cycle is dysregulated and thus, the development of drugs targeting regulators of the cell cycle is a promising strategy. One read-out to screen for such drugs in a high-throughput manner is the Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) system. In this system, the cells show reciprocally oscillating signals of the two fluorescent proteins mAzamiGreen and Kusabira-Orange2 depending on their cell cycle phase. We exchanged Kusabira-Orange2 of the original FUCCI system with a cyan-excitable orange-red fluorescent protein*, which has a long Stoke's shift. Hence, both proteins can now be excited with blue/cyan light making the system suitable for single-excitation dual-emission microscopy. After lentiviral transduction, a stable clonal cell line expressing the novel FUCCI system was generated using imaging with NYONE® Scientific and the Single Cell Cloning (SCC) application of YT®-Software. To prove the functionality of the FUCCI system, we treated the cells with the CDK4/6 inhibitor Palbociclib (PD 0332991) inducing a G1 arrest, or the CDK1 inhibitor RO-3306, which arrests cells in the G2 phase. Subsequently, the cells were imaged in a live-cell imaging manner (Cell Confluence (2F) application) as well as in an endpoint format (Virtual Cytoplasm (2F) application) using NYONE® Scientific and YT®-Software. The modified FUCCI system in combination with SYNENTEC's imagers reliably displayed the expected cell cycle changes. As imaging and analysis is fast and robust, the described system is a powerful method for high-throughput or high-content screening.

KEYWORDS: SINGLE CELL CLONING, FUCCI, CELL CYCLE, PALBOCICLIB, RO-3306, DRUG SCREENING, HIGH-TROUGHPUT SCREENING (HTS), HIGH-CONTENT SCREENING (HCS), CDK INHIBITORS

BENEFITS OF YT®-SOFTWARE APPLICATIONS USED IN THIS NOTE

- The **Single Cell Cloning** applicaton enabled the fast generation of a cell line by limited dilution and the proof of clonality.
- The **Cell Confluence (2F)** application was used to quantifiy the FUCCI signal in living cells over time.
- The **Virtual Cyto (2F)** application quantified the percentage of FUCCI-positive cells using Hoechst staining.

***As this Application Note contains unpublished data, details about the pancreatic cell line as well as the fluorescent protein cannot be disclosed at the time of publication. Therefore, the protein is just called 'FP1' for fluorescent protein 1 throughout this note.**

INTRODUCTION

Cancer is one of the most common reasons for morbidity, and without treatment there is a high mortality [1]. Therefore, it is fundamental to screen for new drugs and to understand their mechanism of action [2]. As many anti-cancer drugs like classical chemotherapeutics target proliferating cells by impacting their progression through the cell cycle [3], analysis of the cell cycle could be an interesting strategy in the screening process. One tool to analyze effects on the cell cycle is the Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) system [4].

The FUCCI system was developed to monitor the transition from G1 to S phase, which cannot be observed by morphological changes (in contrast to the transition from M to G1 phase which is displayed as cell division) [5]. For the visualization of the different phases by fluorescence, two important regulators of the cell cycle were coupled to fluorescent proteins. One of them is geminin (hGem), which is proteolytically degraded from mid-M to G1 phase, meaning it is only active from S to early M phase. This protein was coupled to the fluorescent protein AzamiGreen (mAG). The second protein is Cdt1 (hCdt1), which is proteolytically degraded from S to early M phase, and was coupled to Kusabira-Orange2 (mKO2) in the original FUCCI system. Thus, a reciprocally oscillating fluorescence during the cell cycle is enabled, i.e. during G1 phase the nuclei are red while during S, G2 and early M phase they are green (figure 1) [4], [5].

A limitation of the FUCCI system is the inability to utilize it for broader applications such as deep tissue imaging with multiphoton microscopy. The ability to image thicker tissue specimens, as well as intravital whole organ imaging in small animal models will allow for the same biosensor to be utilized at the cellular, tissue and organ level.

The aim of this study was to develop a FUCCI system suitable for single-excitation dual-emission microscopy (e.g. multiphoton microscopy), in which both fluorescent proteins can be excited with just one wavelength. Moreover, we wanted to confirm the usability of the new FUCCI system as indicator for drug-induced cell cycle arrest at different phases. Among such drugs are inhibitors of Cyclin-dependent kinases (CDKs), which are important cell cycle regulators [6], [7]. We used two of these inhibitors – Palbociclib and RO-3306 [8]. The CDK4/6 inhibitor Palbociclib induces an arrest in G1 phase as CDK4/6 promotes the transition of G1 to S, while the CDK1 inhibitor RO-3306 leads to an arrest in G2/M phase as CDK1 promotes the G2/M phase progression (figure 1) [6], [7]. The aim of this study was to prove that those cell cycle arrests could be observed by our modified FUCCI system.

For imaging of all experiments, we used our automated cell imagers NYONE® Scientific or CELLAVISTA®. These imagers exhibit several advantages to conventional fluorescence microscopes:

- They do not use fixed filter cubes but separate paths for excitation and emission so that each exciter can freely be combined with each emission filter. Thus, also proteins with long Stoke's shifts can be imaged.
- They are designed to image cell culture plates so that the cells do not need to be seeded on microscopic slides for fluorescence imaging and can grow in their usual dishes or plates.
- They contain a fast and robust auto-focus, enabling very fast and easy imaging of the plates.
- They come along with the powerful YT®-Software, which contains many different image analysis applications enabling an easy and robust quantification of the fluorescent signals.
- They can be integrated into pipelines for automation and liquid handling.

Especially due to the potential of automation and high-throughput, the combination of the FUCCI system with our automated imagers could be a very powerful tool for high-content screening in drug development.

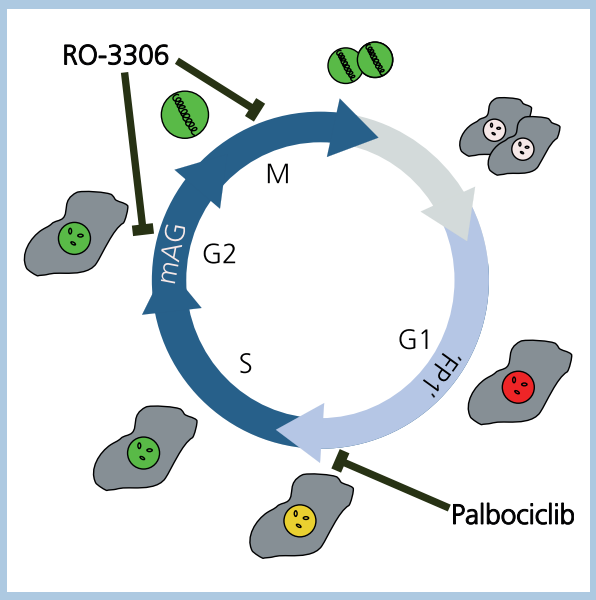


FIGURE 1: OVERVIEW OF THE MODIFIED FUCCI-SYSTEM

The cells express Ctd1-'FP1' and thus show a red fluorescence from G1 to S phase. From S to early M phase, they express geminin-mAzamiGreen and thus show a green fluorescence. The CDK4/6 inhibitor Palbociclib leads to a G1 arrest and the CDK1 inhibitor RO-3306 to an arrest in G2/M phase (based on Sakaue-Sawano et al. [5]).

MATERIAL

- human pancreatic ductal adenocarcinoma cell line
- RPMI1640 medium (e.g. PAN Biotech) supplemented with 10 % (v/v) FCS, 1 % (v/v) L-glutamine, 1 % (v/v) sodium pyruvate, 1 µg/mL puromycin
- FluoroBrite DMEM Media (Gibco Cat. No. A1896701)
- 96 well plates, 48 well plates, 6 well plates, T75 cell culture flask (e.g. Nunc, Greiner)
- Trypsin 0.05 %/ EDTA 0.02 % (e.g. PAN Biotech)
- Palbociclib (CDK4/6 Inhibitor PD 0332991, 8 mM in H_2O, e.g. Sigma-Aldrich Cat. No. PZ0199)
- RO-3306 (CDK1 Inhibitor IV, 10 mM in DMSO, e.g. Sigma-Aldrich Cat. No. SML0569)
- Hoechst33342 (5 mg/mL, e.g. Invitrogen Cat. No. H1399)
- DMSO (e.g. Invitrogen Cat. No. D12345)

METHODS

FUCCI vector construction and production of lentiviruses

We obtained the FUCCI construct mAG-hGeminin from MBL International Corporation. We have combined the sequences of 'FP1' and hCtd1 in silico [5]. 'FP1'-hCtd1 was then synthesized by BioCat (Heidelberg, Germany). Both constructs were sent to Sirion-Biotech, who integrated them into a pCLV-CMV-MCS-IRES-Puro lentiviral vector. Both constructs were expressed under the CMV promoter separated by a 2TA sequence. Sirion-Biotech produced and shipped the functional lentiviruses that we used for transduction.

Cell culture and cell counting

We routinely cultured pancreatic cancer cells in RPMI1640 medium containing FCS (see above) using standardized cell culture conditions (37 °C, 5 % CO_2, humidified atmosphere). Transduced cell lines were routinely cultured in RPMI1640 medium supplemented with FCS (see above) and 1 µg/mL puromycin. Before seeding cells for experiments, we trypsinized the cells and counted them using SYNENTEC's **Trypan Blue** application.

Transduction of pancreatic cancer cells

For the transduction, we seeded 50,000 pancreatic cancer cells per well in a 48 well plate (Sarstedt) and after 24 h, incubated them with RPMI1640 medium containing polybrene and the lentiviruses (MOI 2). After 24 h, we removed the medium and selected the cells in RPMI1640 medium containing 1 µg/mL puromycin. The optimal concentration of puromycin was obtained by establishing a kill curve in preliminary experiments. We changed the medium every two days and observed efficient transduction by imaging the cells in NYONE® Scientific using the respective fluorescence settings for mAzamiGreen and 'FP1'.

Single cell cloning and expansion of clonal colonies

For single cell cloning, we counted transduced and puromycin-selected pancreatic cancer cells after trypsinization. We diluted the cell suspension with medium to a final concentration of 2.5 cells/mL, and seeded the cells at a density of 0.5 cells/well (200 µL/well) in four 96 well plates. The plates were centrifuged (1 min, 30 x g) and measured directly after seeding and further twice a week using the **Single Cell Cloning (SCC)** application of YT®-Software. After one week, we analyzed the fluorescence of the colonies using the **Cell Confluence (2F)** application of YT®-Software. Clonal colonies still showing fluorescence in both channels were transferred to 48 well plates after 2.5 weeks and afterwards to 6 well plates and cell culture flasks. The cells were frozen after passage 3 for the master cell bank.

Determination of the growth rate of the different colonies

For the determination of the growth rate of the colonies in comparison to parental cells, we counted the cells and seeded them in six wells of a 96 well plate (7,500 cells/well). At day one to four and day seven after seeding, the cell confluence was analyzed using NYONE® Scientific and the **Cell Confluence** application of YT®-Software. We exported the data and calculated the growth rate in GraphPad Prism.

Cell cycle inhibition assay

For a cell cycle inhibition (CCI) assay, we seeded 5,000 cells/well in a black, clear and flat-bottom 96 well plate. At day one after seeding, the plate was directly measured before treatment with Palbociclib (8 µM), RO-3306 (9 µM) or DMSO as control and after the treatment at different time points (0 h, 2 h, 4 h, 6 h and 24 h) using SYNENTEC's **Cell Confluence (2F)** application. We exported the data of the processed experiments from YT®-Software and calculated the difference of the cell area showing a fluorescent signal between the treated and the control cells using equation 1 (Fluo = Fluorescent area, parameter "BF and Fluo Area / BF Area [%]"). After 24 h, we added Hoechst33342 to the medium and incubated the cells for 20 min in the incubator. We exchanged the medium to FluoroBrite medium and measured the plate using SYNENTEC's **Virtual Cytoplasm (2F)** application. We exported the data of the processed experiments from YT®-Software and calculated the difference of the positive cells between the treated and the control cells using equation 2 (PosCells = % positive cells, parameter "TC-nn/np/pn,pp [%]") Graphs were generated in GraphPad Prism.

Equation 1

$$\Delta Fluo = Fluo - \overline{Fluo}_{control}$$

Equation 2

$$\Delta PosCells = PosCells_{treated} - \overline{PosCells}_{control}$$

TABLE 1: IMAGING SETTINGS FOR THE MODIFIED FUCCI-'FP1'-mAG SYSTEM

Channel	Excitation LED [nm]	Emission Filter [nm]
Brightfield	Brightfield	Green (530/43)
mAG-hGeminin	Blue (475/28)	Green (530/43)
'FP1'-hCtd1	Blue (475/28)	Red-LP (593-LP)

RESULTS & DISCUSSION

1. Cloning of the FUCCI-'FP1' vector and lentiviral transduction

To use the FUCCI system for single-excitation dual-emission microscopy, we modified the original FUCCI system by replacing mKO2 by 'FP1', which has a long Stoke`s shift. 'FP1' can thus be excited with the same wavelength as mAG but emits orange light. After lentiviral packaging, we transduced a pancreatic cancer cell line with this modified FUCCI system. We were able to image both fluorescence signals in NYONE® Scientific using the settings described in table 1. We expanded cells showing an efficient transduction in puromycin-containing selection medium.

2. Limited dilution and proof of single cell clonality

To generate and expand a clonal cell population, we seeded transduced pancreatic cancer cells at a density of 0.5 cells/well. The plates were imaged and analyzed directly after seeding and at regular intervals using the **SCC** application of YT®-Software. Once colonies had formed, they were detected by the **SCC** application (figure 2 A). By going back to the first measurement, we could confirm the expansion from a single cell (figure 2 B).

After the formation of the colonies, plates were imaged using the imaging settings described in table 1 to confirm that both fluorescent proteins were still expressed (figure 2 C). We

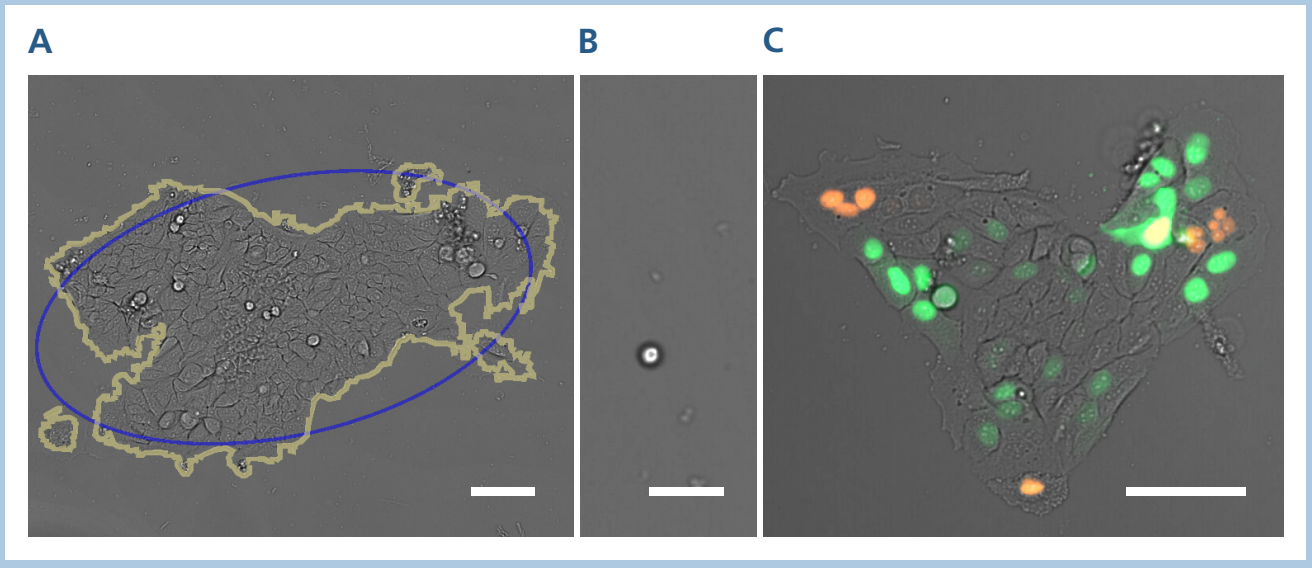


FIGURE 2: THE SCC APPLICATION CONFIRMED THE CLONALITY OF THE TRANSDUCED CELL LINE, AND THE CONFLUENCE 2F APPLICATION ENABLED VISUALIZATION OF THE EXPRESSION OF THE MODIFIED FUCCI SYSTEM

We seeded 0.5 cells/well in a 96 well plate and imaged the microplate twice a week over 2.5 weeks starting at day 0. A) YT®-Software detected the colony (day 14, cell confluence is labelled by a yellow line and the colony by a blue ellipse, 10 x objective, scale bar = 100 µm). B) To confirm the monoclonality of the detected colony, the measurement immediately after seeding is viewed to see if a single cell is visible (day 0, 10 x objective, scale bar = 50 µm). C) Fluorescent visualization of the colony after one week shows green and orange/red signals indicating the expression of the FUCCI system (day 9, 10 x objective, scale bar = 100 µm).

transferred and expanded only clones derived from a single cell with confirmed fluorescence. By this procedure and verification, eight clonal cell lines were generated.

3. Comparison of the growth of transduced cell lines to the parental cell line

To ensure, that the transduced cells behave similar to the parental cells, we compared their growth curves using the **Cell Confluence (2F)** application of YT®-Software.

YT®-Software automatically depicts Time Charts in the Analyst Tab and thus enables a fast overview of the results for the user (figure 3 A). Moreover, we exported the data ('Cell Confluence BF') and calculated growth curves using logistic growth in the statistic software GraphPad Prism (figure 3 B). For the calculation of the growth rates, the logistic growth equation 3 was used with k = growth rate, $Confl_{max}$ = maximum confluence of 100 % and $Confl_0$ = starting confluence (table 2).

Equation 3

$$Confl(t) = Confl_{max} \cdot Confl_0 / [(Confl_{max} - Confl_0) \cdot e^{-k \cdot t} + Confl_0)]$$

The growth behavior of the different clonal cell lines were slightly different compared to the parental cells. We decided to use clone 1704C4 for further experiments as it had the most similar growth rate as the parental cells (figure 3 C).

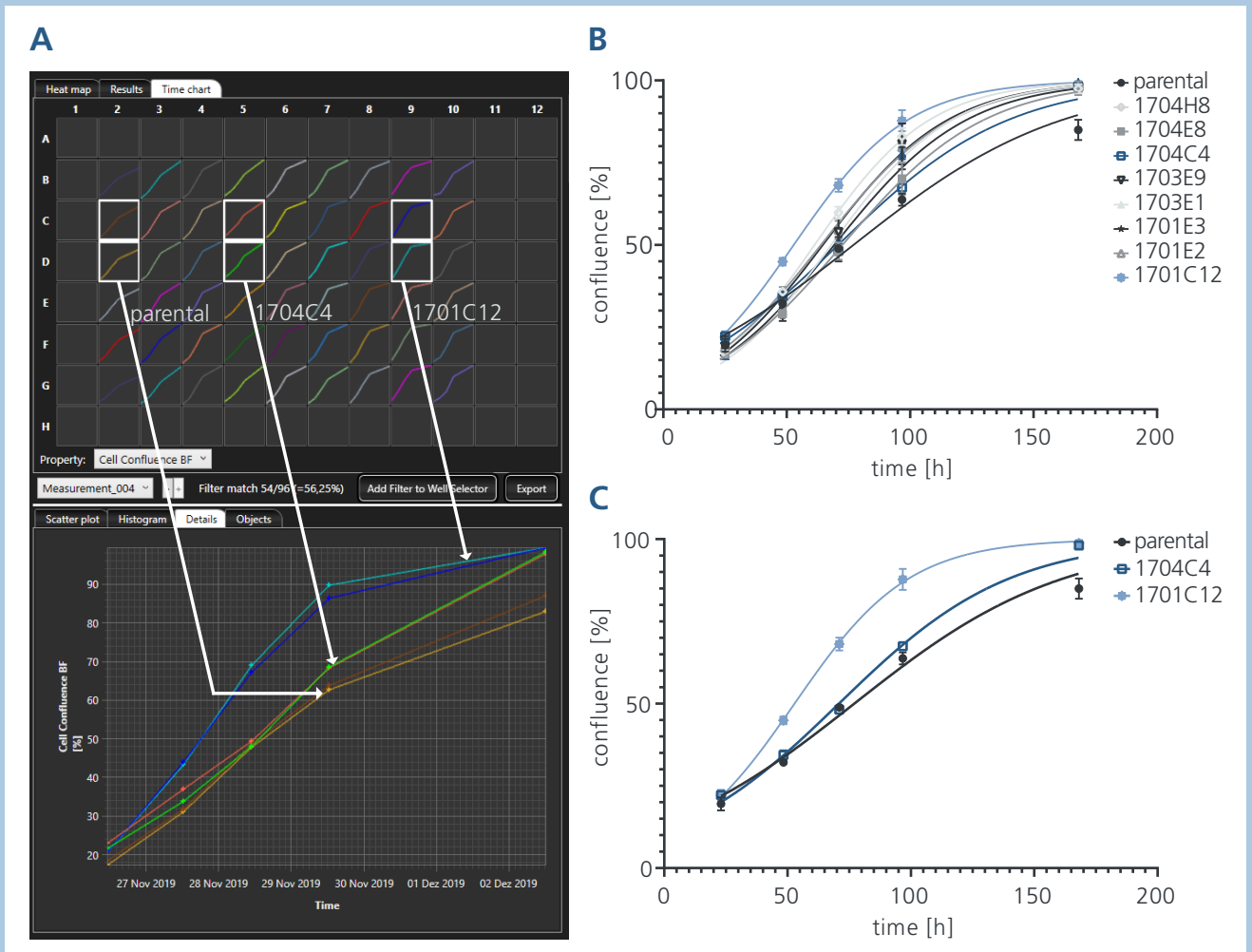


FIGURE 3: GROWTH CURVES OF TRANSDUCED AND PARENTAL CELLS CAN BE COMPARED USING THE CONFLUENCE APPLICATION OF YT®-SOFTWARE

We seeded cells of each clonal cell line in six wells of a 96 well plate and monitored the cell confluence using NYONE® Scientific. A) Screenshot of the *Time Chart* as observed in the Analyst Tab in YT®-Software. As an example, parental cells in row two, clone 1704C4 in row five and clone 1701C12 in row nine were selected and the growth curve over a five-day period is shown below. B) We exported the results ('Cell Confluence') and calculated the growth curve of all cell lines using the logistic growth equation of the statistic software GraphPad Prism (mean of 'Cell Confluence' [%] + SD, n = 6). C) Same data as in B, but for better visualization, only clone 1701C12 showing a faster cell growth and clone 1704C4 showing a similar growth curve to the parental cells are shown. Clone 1704C4 was used for further experiments (mean of 'Cell Confluence' [%] + SD, n = 6).

TABLE 2: GROWTH RATES OF PARENTAL CELLS AND CELLS TRANSDUCED WITH THE MODIFIED FUCCI-'FP1'-mAG SYSTEM

Clone	parental	1704H8	1704E8	1704C4	1703E9	1703E1	1701E3	1701E2	1701C12
Growth rate k [h^{-1}]	0.02355	0.04280	0.03478	0.02891	0.03939	0.04072	0.03719	0.03779	0.04310

4. Functional validation of the modified FUCCI system

Beside the growth behavior, we validated the functionality of the FUCCI system. At first, we seeded the cell line 1704C4 in a 96 well plate and monitored the change of fluorescence over time (figure 4).

The monitored cells were changing their fluorescence from red to yellow to green or from green to colorless to red during this time (population doubling time of approximately 24 h). As an example, in the first row in figure 4, a cell directly before mitosis is depicted showing green fluorescence. In a functional FUCCI system, this would be expected as hGem-mAG should be active in this cell cycle phase while hCtd1-'FP1' should be degraded (figure 1). After mitosis, the daughter cells lose their fluorescence, which is also in accordance with a functional FUCCI system. After several hours, an orange/red signal arises in both daughter cells, indicating the expression/stabilization of hCtd1-'FP1'. In the second row, two cells have the red fluorescence of mAG. The activity of hGem-mAG indicates that the cells are in G1 phase after mitosis. The start of the activity of hCtd1-'FP1' upon entry into the S phase results in a color shift from red to yellow. Only after degradation of mAG the cells have green fluorescence. The cells in the last row also show this behavior of color change from colorless to red via yellow to green.

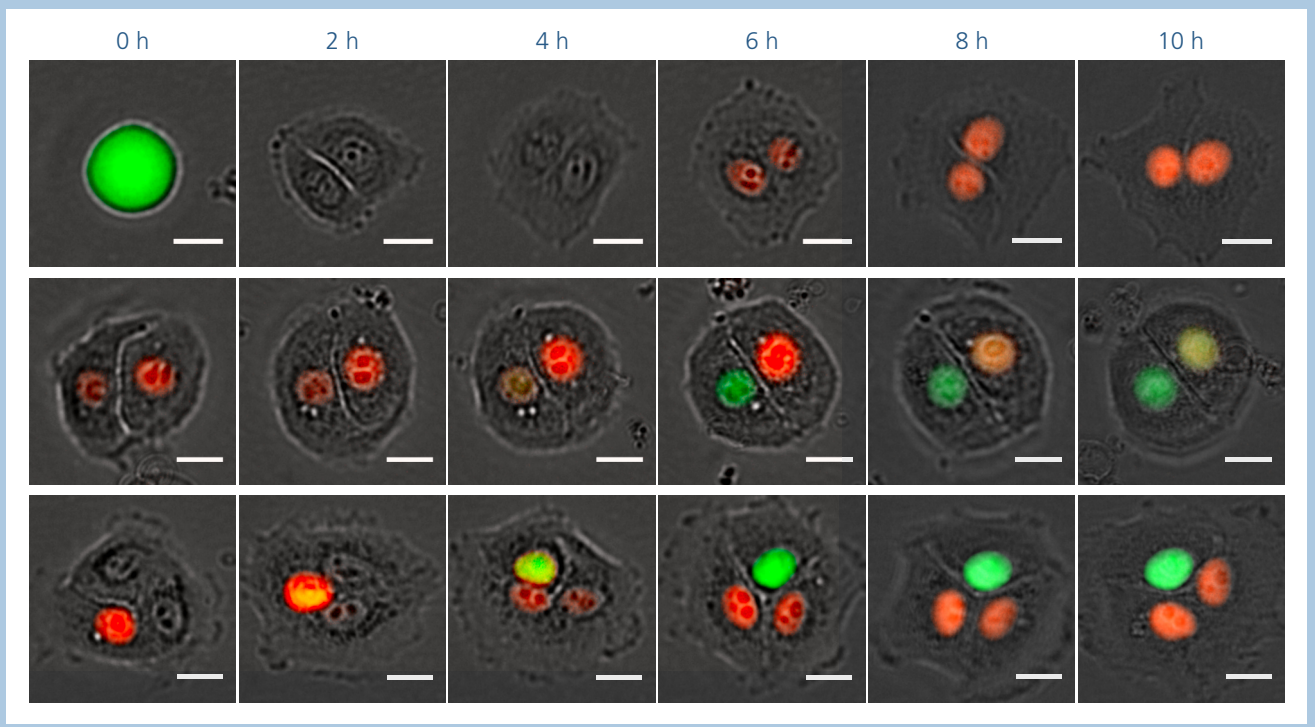


FIGURE 4: VALIDATION OF A FUNCTIONAL FUCCI SYSTEM

We seeded cells of the transduced clonal cell line 1704C4 in a 96 well plate. After 24 h, we imaged the cells at the indicated time points using NYONE® Scientific. The fluorescent signals were changing during the observation time. Representative cells displaying characteristic shifts in their fluorescence are shown corresponding to the expression of Ctd1-'FP1' (red fluorescence) from G1 to S phase and hGem-mAG (green fluorescence) during S to G2 phase (scale bar = 15 µm).

To demonstrate, that the modified FUCCI system also indicates drug-mediated arrests in different cell cycle phases, we treated the cells with the two CDK inhibitors Palbociclib (CDK4/6 inhibitor inducing a G1 arrest) and RO-3306 (CDK1 inhibitor inducing a G2/M arrest). During treatment, we imaged the cells using SYNENTEC's **Cell Confluence (2F)** application. This allowed for the determination of the area of the respective fluorescent signal within a cell area in relation to the overall cell area (figure 5 A). After Palbociclib treatment, the area of the green fluorescence (S/G2/M phase) decreased in comparison to the control cells, while after RO-3306 treatment it increased (figure 5 B). As expected, the converse was depicted for the red fluorescence (G1 phase) (figure 5 C).

To determine the percentage of green and/or red fluorescent cells, at the end of the 24 h treatment with CDK inhibitors, we added Hoechst33342 to the cells. Thereby, the nuclei of the cells could be counted and the expression of the FUCCI proteins evaluated using SYNENTEC's **Virtual Cytoplasm (2F)** application. This application allows detection of the Hoechst-stained nuclei (all cells), and recognizes if the nucleus contains also orange, green or no fluorescence (figure 6 A+B). We exported the data of the processed experiments from YT®-Software and calculated the difference of the CDK-inhibitor- and the DMSO-treated cells (figure 6 C). As expected, the percentage of cells in G1 or S/G2/M phase drifted corresponding to the treatment with the inhibitors.

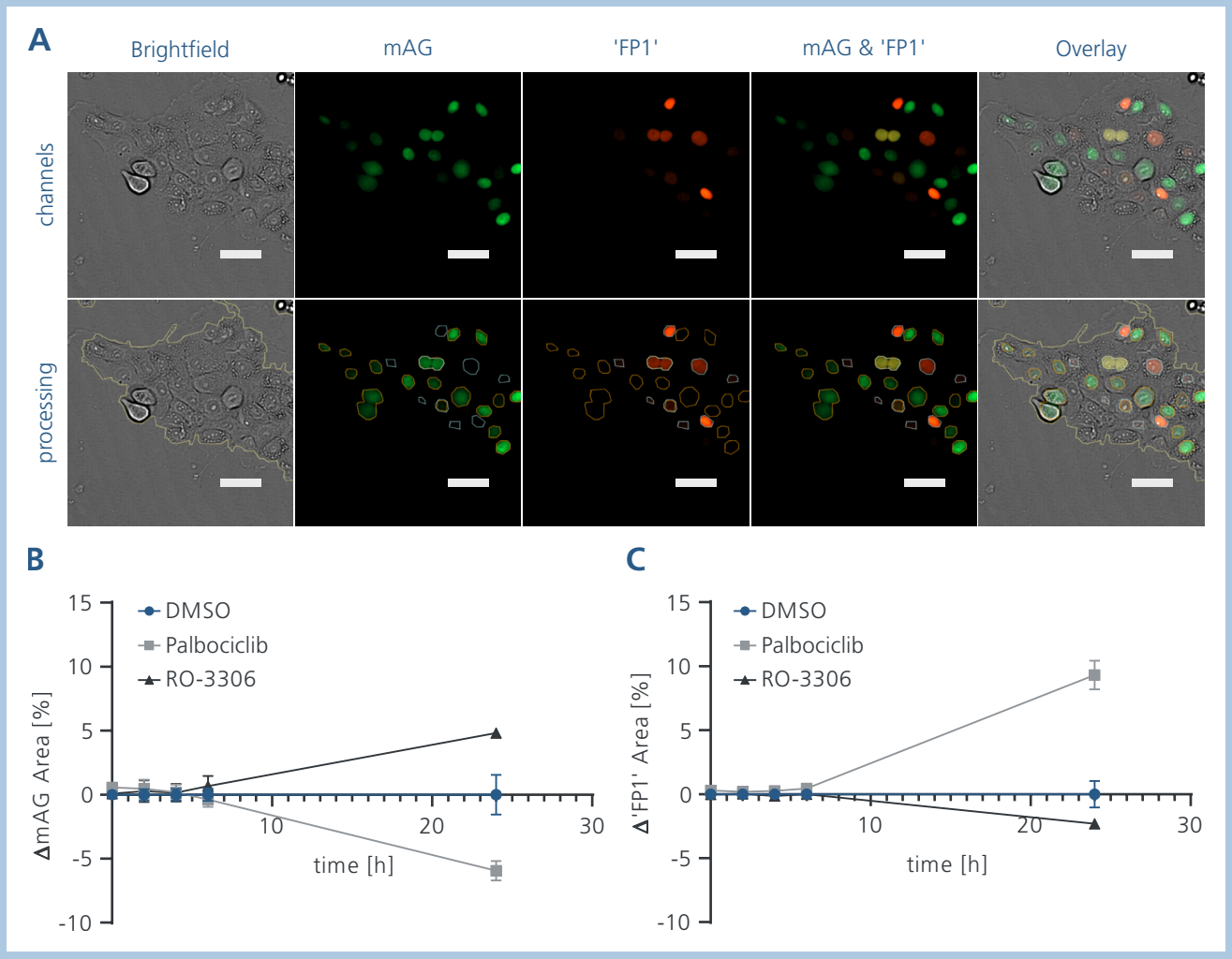


FIGURE 5: CELL CYCLE ARRESTS INDUCED BY CDK INHIBITORS WERE OBSERVED OVER TIME USING THE CELL CONFLUENCE (2F) APPLICATION OF YT®-SOFTWARE

For a cell cycle inhibition (CCI) assay, we seeded cells of FUCCI-transduced clonal cell line 1704C4 in a 96 well plate, treated the cells for 24 h with 8 µM Palbociclib, 9 µM RO-3306 or DMSO as control and imaged the cells at different time points with NYONE® Scientific. A) Cells were imaged and the images were automatically processed using the Cell Confluence (2F) application of YT®-Software. The image processing labels the confluent area with a yellow line, while green and orange/red areas are encircled orange or light blue, respectively (scale bar = 50 µm). B) We exported the data and analyzed them using GraphPad Prism. The difference of the green fluorescent area (mAG) of treated cells to control cells over time is shown (mean ΔmAG Area [%] + SD, n = 6). C) The difference of the red fluorescence ('FP1') area over time is shown (mean ΔFP1 Area [%] + SD, n = 6).

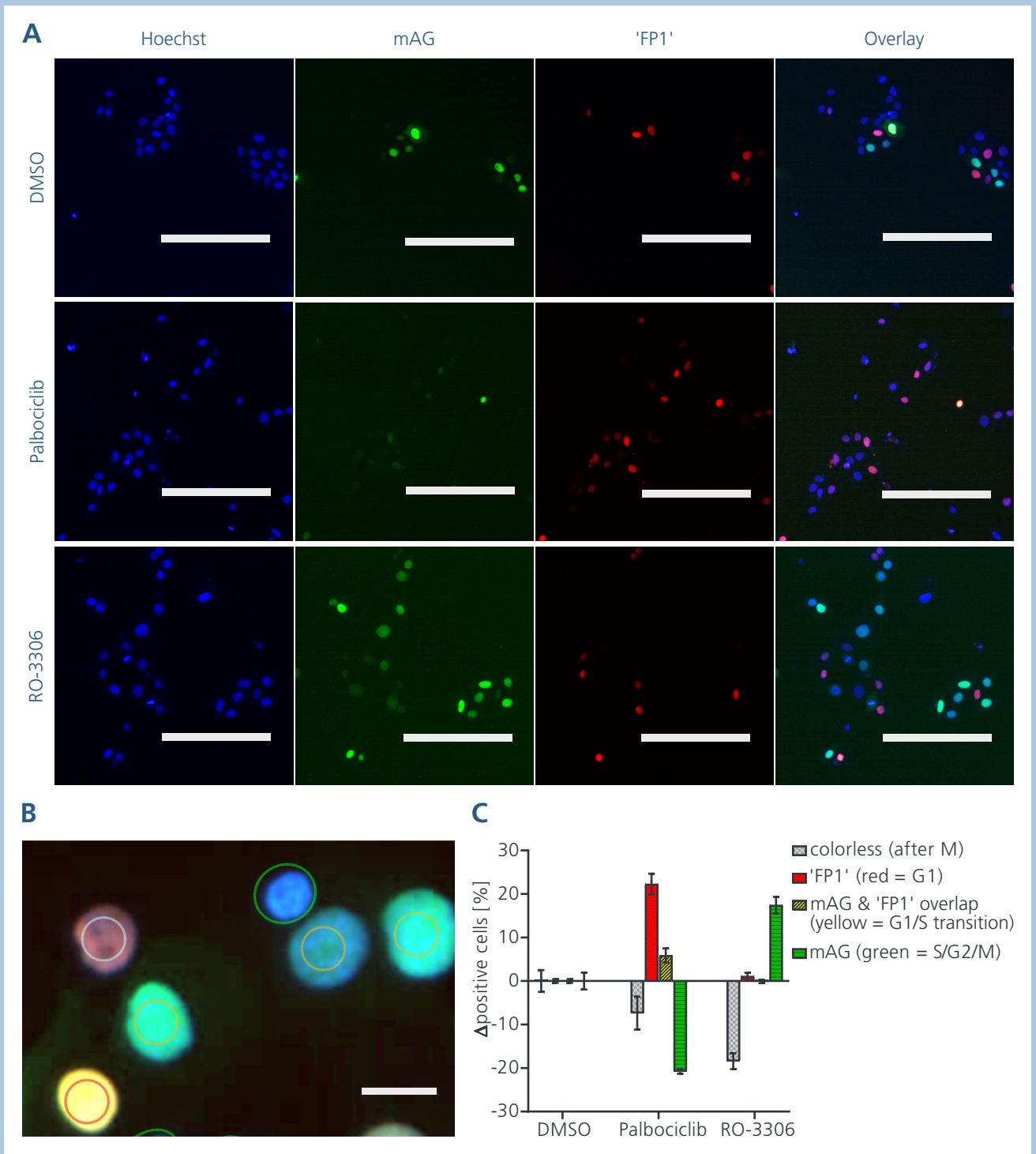


FIGURE 6: CELL CYCLE ARRESTS INDUCED BY CDK INHIBITORS WERE OBSERVED USING THE VIRTUAL CYTOPLASM (2F) APPLICATION

For the cell cycle inhibition (CCI) assay, we seeded cells of the clonal cell line 1704C4 in a 96 well plate, treated the cells for 24 h with 8 µM Palbociclib, 9 µM RO-3306 or DMSO as control and added Hoechst33342 before imaging with NYONE®. A) The DMSO-, Palbociclib- and RO-3306-treated cells were imaged in the different channels. Representative images are shown (scale bar = 200 µm). B) Image processing of the Virtual Cytoplasm (2F) application detects the Hoechst-positive nuclei and analyzes the green or orange fluorescence within it. Nuclei that are non-fluorescent in both channels are marked with a green line, mAG-positive ones by an orange circle, 'FP1'-positive ones by a light blue circle and double positive ones by a red circle (scale bar = 20 µm). C) The result of the CCI assay as evaluated by SYNENTEC's Virtual Cytoplasm (2F) application were exported and evaluated with GraphPad Prism (equation 1). The difference of the percentage of positive cells to the positive cells of the control-treated cells is shown (mean of Δpositive cells [%] + SD, n = 6).

CONCLUSION

We generated a clonal pancreatic cancer cell line (clone 1704C4) with a modified FUCCI system indicating the different cell cycle phases. In the analytical process, SYNENTEC's imagers NYONE® Scientific or CELLAVISTA® proved to be valuable tools for the following reasons. Firstly, the **SCC** application is a fast and precise method for the reliable generation of clonal cell lines in a high-throughput manner. Secondly, the growth characteristics of the generated cell lines could be analyzed directly in a very efficient way using the **Cell Confluence** application. Finally, the functionality of the FUCCI system was demonstrated using the **Cell Confluence (2F)** application allowing real-time live cell imaging or the **Virtual Cytoplasm (2F)** application, providing an accurate determination of the percentage of fluorescent cell nuclei. In summary, we have generated a modified FUCCI biosensor to monitor cell cycle changes and validated the system using SYNENTEC's imagers with their unique YT®-Software. SYNENTC's imagers allow for precise HTS/HCS analyses and combined with the modified FUCCI system are valuable tools to advance our understanding of disease pathology and to develop new drugs.

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