Live quantitative monitoring of mineral deposition in stem cells using tetracycline hydrochloride.

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Abstract

The final stage of *in vitro* osteogenic differentiation is characterized by the production of mineral deposits containing calcium cations and inorganic phosphates, which populate the extracellular matrix surrounding the cell monolayer. Conventional histological techniques for the assessment of mineralization, such as Von Kossa and Alizarin Red S staining, are end-point techniques requiring cell fixation. Moreover, in both cases staining quantitation requires dye extraction which irreversibly alters the ECM conformation and structure, therefore preventing the use of the sample for further analysis.

In this study, the use of Tetracycline hydrochloride (TC) is proposed for the non-destructive staining, quantitation and imaging of mineralizing bone-like nodules in live cultures of human bone marrow mesenchymal stem cells (MSCs) cultured under osteogenic conditions. Overnight administration of TC to living cells was shown not to alter the metabolic activity or the progression of cell differentiation. When applied to differentiating cultures, cell exposure

to serial doses of TC was found to produce quantifiable fluorescence emission specifically in osteogenic cultures. Incubation with TC enabled fluorescence imaging of mineralised areas in live cultures and the combination with other fluorophores using appropriate filters. These results demonstrate that serial TC administration over the differentiation time course provides a qualitative and quantitative tool for the monitoring and evaluation of the differentiation process in live cells.

Running Title: Live & quantitative monitoring of stem cell mineralisation.

Keywords: stem cells, osteogenesis, in vitro differentiation, live cell imaging, quantitative assay.

Introduction

The cellular process underpinning bone formation is routinely modelled *in vitro* using different types of cells including primary osteoblasts, multipotent and pluripotent stem cells which, under specific physico-chemical stimulation, differentiate into mineralizing bone-like cells [1]. During osteogenic differentiation, a well-organized and collagen-enriched extracellular matrix is formed followed by the production of extracellular minerals deposits made of calcium and inorganic phosphates [2]. In conventional 2D culture systems, these minerals deposits can be identified on top of the cell monolayer through brightfield imaging [3]. However, qualitative and quantitative assessments of mineralization are essential for the full characterization of differentiation, and are conventionally achieved with histological stain techniques. The most widely used assays include Von Kossa and Alizarin R staining, which respectively target anionic phosphates and calcium cations [4, 5]. However, both methods are end-point assays and require cell fixation, followed by multiple staining steps. To overcome these limitations and enable live analyses, diverse fluorochrome-based labelling methods have been proposed for the evaluation of bone formation *in vitro* and *in vivo* such us Giemsa, Calcein blue and Xylenol Orange among others [6-9].

Tetracyclines (TCs) constitute a wide family of broad spectrum antibiotics classified as natural, semisynthetic and chemically-modified according to their origin [10] . In addition to their antimicrobial activity, these compounds are characterized by their calcium chelating ability and fluorescence emission [11, 12]. These properties have led TCs to be used as a marker of calcification front in bone, applied *in vivo* by parenteral or enteral administration, or used for staining bone biopsies post fixation [13]. TCs have also more recently been used for the qualitative observation of mineralized ECM in dental pulp cells culture by fluorescence

imaging [14]. However, TCs have not yet been used for the quantitative evaluation of mineralisation in live cultures.

The aim of this study was to investigate the use of a Tetracycline for the non-destructive *in vitro* staining, quantification and live imaging of bone-like mineralized ECM using differentiating human mesenchymal stem cells.

Material and methods

All reagents were purchased from Thermofisher (UK) unless otherwise stated.

1. Cell culture and differentiation

Immortalized human bone marrow-derived mesenchymal stem cells (MSCs) [15-17] were seeded at a density of 4000 cells/cm^2 in 48-well plates in standard culture medium (SC) (low glucose DMEM supplemented with 10% foetal calf serum, 1 % penicillin and streptomycin, 1% L-Glutamine, 1% of non-essential amino acids). After 24 hours, the standard medium was replaced with osteogenic medium (OS) (standard medium supplemented 0.1 µM Dexametasone, 10 mM β-glycerophosphate, and 50 µM ascorbic acid (Sigma-Aldrich, UK)) to induce osteogenic lineage differentiation [18]. Cells were cultured for 21 days at 37 ^{o}C and 5% CO_2, with medium refreshment every 48 hours.

2. Evaluation of cytotoxicity

Cell metabolic activity was analysed using Presto Blue reagent at day 7, day 14 and day 21, according to the manufacturer's instructions. Briefly, the cells were washed once with PBS and incubated for 40 minutes with 300 µl standard medium containing 10% of Presto Blue

reagent at 37 ^{o}C. 250 µl were transferred to a new 96-well plate and the fluorescence was measured in a microplate reader (Tecan Infinite 200) using excitation and emission wavelengths set at 560 nm and 590 nm, respectively.

3. ALP assay and Alizarin Red S staining

Alkaline phosphatase (ALP) activity was assayed at day 7 and day 14 of culture. A solution containing 1 mg/ml p-nitrophenyl phosphate and 0.2 M Tris buffer (SIGMAFAST, Sigma-Aldrich) was prepared according to the manufacturer's instructions. Cells seeded in 48-well plates were washed twice with PBS, and 300 µl of assay solution were added to each well. ALP activity was monitored by performing 12 readings of the optical density at 405 nm over 24 min in a microplate reader. Cells were then washed twice and fresh medium was added before returning the cells to the incubator until the following time-point.

Alizarin Red S staining was performed at day 7, day 14 or day 21 as stated. Cell fixation was performed using 4 % paraformaldehyde for 10 min at 4 ^{o}C. Before the staining, fixed cells were washed twice with deionized water. Then, 200 µl of 1 % w/v Alizarin Red S solution (Sigma-Aldrich) were added to each well for 10-15 min, followed by extensive washing with deionized water before imaging. For Alizarin Red staining quantification, stained cells were washed with deionized water and incubated with 200 µl of destaining solution (20 % methanol, 10 % acetic acid in deionized water) during 15-20 min before measuring the absorbance of the solution at 405 nm in the microplate reader.

4. Tetracycline administration and analysis of fluorescence emission

The tetracycline (TC) staining solution chosen was prepared with Tetracycline Hydrochloride (Sigma-Aldrich) dissolved in PBS, filtered through 0.22 µm syringe filter, and administrated to

the cells at final concentrations of 5 µg/ml, 10 µg/ml, 20 µg/ml or 40 µg /ml in culture medium at day 6, day 13 and day 20. After 18 to 22 hours of incubation with TC, cells were washed twice with PBS; then, 300 µl of PBS were added to each well and fluorescence was measured using the microplate reader (Tecan infinite 200), recording 25 reading points *per* wells at 390 nm excitation and 560 nm emission.

5. Cell staining and imaging

For both live and fixed samples, cell nuclei were counterstained by incubating cells with 10 µg/ml of Hoechst 33258 for 10 min. Cytoskeletal actin fibres were visualised in fixed cells using Vectashield mounting medium containing TRITC-Phalloidin (Vector Laboratories, UK). Live cells were imaged on a Leica DM IRB microscope using the A filter cube (excitation filter λ 340-380 nm, emission long pass filter λ 425 nm) and coupled to a QICAM Fast 1394 camera. Confocal laser scanning microscopy was carried out on a Zeiss Elyra PS.1 microscope equipped with LSM 780 confocal unit using 10x/0.45NA water immersion objective. The TC emission spectrum was acquired in Lambda mode using a 32 channel meta-detector in the confocal microscope. After inspecting the emission signals, sequential imaging channels were set for each fluorophore, and for each channel the respective laser was assigned: Hoechst: $\lambda_{ex.}$ 405 nm laser, $\lambda_{em.}$ 405-437 nm; TC: $\lambda_{ex.}$ 405 nm laser, $\lambda_{em.}$ 588-650 nm; TRITC-Phalloidin: $\lambda_{ex.}$ 561nm laser, $\lambda_{em.}$ 579-641 nm.

6. Statistics

Three independent experiments were performed;; results are presented as mean ± SEM. One-way ANOVA with Tukey's multiple comparison post hoc test was used. A 95% confidence

level was considered significant. Statistical analysis was performed with the GraphPad PRISM 7.01 software package.

Results

1. Tetracycline as an in vitro live stain for osteogenic differentiation

In order to test the ability of TC to stain mineralising cells in culture and determine the optimal working concentration then differentiated and undifferentiated MSCs cultured for 20 days *in vitro* were incubated for 18 to 22 hours with four TC concentrations. Live fluorescence imaging revealed a green staining pattern that co-localized with the grainy mineral deposits visible on the top of the cell monolayer in brightfield mode. The signal was stronger and more defined as the TC concentration increased, while no staining was detected in either differentiated cells unexposed to TC or in undifferentiated cells treated with 40 µg/ml of TC (Fig. 1A). Alizarin R staining performed at day 21 in post-fixed cells confirmed the differentiated phenotype of cells transiently exposed to the four concentrations of TC, and showed no significant differences between all treatment groups (p= 0.47), confirming TC exposure did not interfere with the staining (Fig. 1A, B). Metabolic activity measured in cells treated with increasing TC concentrations (Fig. 1C) also showed no differences (p= 0.77), confirming that the TC treatment was non-toxic at all concentrations tested in this study.

The fluorescence intensity observed in TC-treated cultures was measured using a microplate reader and TC signal was significantly higher in cells treated with osteogenic condition than in undifferentiated cells maintained in SC medium (Fig. 2). The 40 µg/ml concentration

resulted in significantly higher values in comparison to all the other concentrations in both culture conditions (Fig. 2).

2. Tetracycline fluorescence spectrum and co-staining with other fluorescent dyes

In order to define the optimal imaging settings for TC cell labelling, the lambda mode of the confocal microscope was used with 405 nm, 488 nm, 561 nm or 633 nm lasers to detect the whole TC spectra in fixed cells treated with 20 µg/ml of TC. Strong emission was detected when a 405 nm laser was used with two major peaks at 520 nm and 584 nm. Importantly, relatively low signals (< 8%) were observed in the emission spectra acquired at other wavelengths (488, 561 and 633 nm), suggesting little or no spillover (Suppl. Fig. 1). To test the possibility of using TC concomitantly with other fluorescent dyes, fixed cells treated with 20 µg/ml of TC where co-stained with Hoechst 33258 and TRITC-Phalloidin to label nuclei and cytoskeleton respectively. Notably, using the 405 nm laser, a residual Hoechst 33258 signal was detected at 520 nm but not at 584 nm. Therefore, the TC imaging protocol was optimised using the 405 nm laser with detection in the range of 588-650 nm, providing a specific TC signal without any spillover signal from Hoechst 33258 or Phalloidin-TRITC fluorophores (Fig. 3).

3. Live Tetracycline staining in differentiating cells

To further optimise TC staining for live monitoring of osteogenic differentiation, TC administration to live cultures was performed in either single or serial doses (20 µg/ml) at day 6, day 13 and day 20 (Fig. 4A), in order to evaluate the biocompatibility and efficiency of multiple TC exposures. Cells exposed to single or repeated TC treatment were first observed

under fluorescence microscopy to evaluate the nature and distribution of the staining over time (Fig. 4B). Live imaging revealed visible TC-stained mineral deposits at day 14 and day 21 of OS treatment, but not at day 7, suggesting minimal differentiation at this time point. At day 14, the TC staining pattern and signal intensity were similar in cells exposed to single and serial TC doses. At day 21 of differentiation however, a sharper and more defined signal was observed after serial administration of TC (3 consecutive doses) in comparison to a single administration, suggesting a cumulative effect of serial TC treatment from day 14 but not from day 7.

Quantification of TC fluorescence was performed in living cells at day 7, day 14 and day 21 of OS differentiation to complement the microscopy observation, confirming time-dependent increase in TC signal (Fig. 5A). The administration of serial doses resulted in significantly higher TC fluorescence intensity at day 21 in comparison to the single dose administration protocol.

When analysing cellular parameters, TC exposure was not found to negatively affect metabolic activity after either single or serial doses at any of the time points analysed (Fig. 5B), and measurements of ALP activity at day 7 and day 14 showed comparable levels in cells after single or serial TC treatment and cells unexposed to TC (Fig. 5C). The Alizarin Red S staining performed confirmed the progression of cell differentiation from day 7 to day 21 (Suppl. Fig. S2). Importantly, staining quantification (Fig. 5D) revealed no significant differences in mineral deposition at any time point between untreated and TC-treated cells, whether using single or serial doses, indicating that TC treatment did not interfere with the progression of cellular differentiation ($p > 0.9999$).

Discussion

The use of *in vitro* live cell assays permits the monitoring and evaluation of cell status in on-going cultures, allowing longitudinal assessment of cellular responses in real-time. In the field of bone research and tissue engineering this is important as *in vitro* studies involve long-term experiments to allow for extracellular matrix maturation and mineralisation, which are typically considered over 3 weeks of cell culture. The assessment of the alkaline phosphatase enzymatic activity in living cells allows for the evaluation of the status of the cell differentiation in real time during the *in vitro* culture period; however, this targets the early stage of differentiation [2]. Moreover, molecular reporter systems engineered using osteogenic gene promoters to drive a reporter marker can allow for a direct follow-up of gene expression and differentiation profile [19], however these imply genetic manipulations therefore preventing their direct application in primary cells. To overcome these limitations, we have developed a new protocol for the non-destructive measurement of mineralization in live cultures using Tetracycline hydrochloride. We have also defined the optimal regime of TC administration to enable staining, quantification and fluorescence imaging in live cells using human mesenchymal stem cells (MSCs) as a differentiation model. We have also showed that TC staining can be multiplexes with other fluorophores to enable advanced cellular analysis of osteogenic cultures.

Among the several tetracycline-derivatives available and already in use for *in vivo* bone labelling, we selected Tetracycline Hydrochloride (TC) as it displayed the highest brightness in comparison to others when used to label rat bone samples [20]. Our results showed that TC administrated in vitro as single or repeated doses did not alter the metabolic activity of human MSCs. The results of several studies suggest that different administration protocols and/or

TC-derivatives may differently affect cell health, and also that sensitivity to TC-derivatives might be cell type-dependent. Indeed, MG-63 human osteosarcoma cells experienced a decrease in number after overnight incubation with 10 µg/ml Doxycline and a significant reduction of proliferation after daily treatment with the same dose of the compound [21]. The treatment of primary human osteoblasts with 190 µg/ml of TC resulted in a 30% increase of LDH, which was considered as an indication of impaired mitochondrial function, and suggested that this cytotoxic effect could be partially mediated by an alteration of mitochondrial respiration [22]. Another study reported that the administration of TC-analogues (Doxicycline, COL-3 and Minocycline) from 10 µg/ml to the acute myeloid leukaemia cell line HL60 reduced cell viability of more than 50 %. By contrast, 1 µg/ml of Doxicycline and Minocycline continuously added to the cell culture medium have been reported to significantly increase the proliferation of human osteoblastic bone marrow cells [23, 24]. Our results also showed that TC administration at day 7 and day 14 during the culture period did not interfere with the induction and progression of the differentiation process. For both alkaline phosphatase activity and mineral deposition assays, no significant differences were observed between cells treated with single and multiple doses of TC in comparison to cultures unexposed to TC. Here too, existing reports on the effect of TC treatment on bone differentiation are contradictory and might depend on the TC-derivative used, the dose and the administration regime. *In vitro*, the daily administration of 1 µg/ml of Doxycycline or Minocycline seemed to significantly promote the mineralisation of human bone marrow-derived osteoblasts [24]. *In vivo*, several studies performed on diverse disease models (such as diabetes, osteopenia and osteoporosis) in mice and rats reported a positive association between the administration of TC-derivatives and bone formation and density [25-28]. However, it has been proposed that such an effect observed *in vivo* might be mediated by an inhibitory

effect of TC on osteoclasts function [28, 29]. As an opposite trend, TC administration at early stages of development or to *ex vivo* embryonic bones, bone growth was compromised followed by increase in bone fragility [30, 31].

The present study also demonstrated that TC is a suitable compound for the evaluation of osteogenic differentiation in living cells such as MSCs, over an extended culture period. All the TC doses tested here (5-40 µg/ml) were selected within the same range as previously reported [14, 31] and were suitable for the fluorescent labelling of mineralising cells, resulting in a more defined signal as the concentration increased. This trend was confirmed by spectrophotometric analysis, which revealed a linear increase of the detected fluorescence signal. However, among the TC doses tested here, 20 µg/ml was selected as optimum to monitor the progression of MSCs differentiation at various time points. While the highest dose (40 µg/ml) provided a more defined and brighter signal in fluorescent images, the spectrophotometric quantitation also showed a significant increase of the signal in undifferentiated cells, suggesting the increase of unspecific background signal at this concentration. In our study, TC labelling was clearly visible as green stain using a conventional DAPI long pass filter at day 14 and day 21, but not at day 7 of osteogenic treatment. This result was in agreement with the spectrophotometric quantitation of TC fluorescence signal and was also confirmed by parallel Alizarin red staining, which did not reveal any detectable mineral deposits at day 7. Due to the calcium tropism of TC, the presence of calcium phosphate minerals in the ECM is necessary for the staining. The production of mineralized ECM is a late event during bone-lineage differentiation, starting around day 10-12, while the earlier period is characterized by active cell proliferation and production of ECM components [2]. Serial TC administration resulted in a stronger and more defined staining in MSCs cultures, particularly at day 21, in comparison to the single dose administration. This result, observed

by microscopy and confirmed by spectrophotometry, suggests the retention and accumulation of the dye into the mineralizing bone-like nodules between the different administrations over the 21 days of culture. This appears in agreement with *in vivo* studies reporting the retention and visualization of this compound in bones up to 4 weeks after administration [32]. These results confirm the suitability of TC staining to monitor the progression of live MSCs differentiation at various time points, using a simple and cell neutral protocol.

The real time evaluation of mineralising cultures has previously been achieved by the continuous administration of Calcein without affecting the cell viability or the progression of differentiation, resulting in a green labelling visible through a FITC long pass filter [8]. However, this method is based on the uptake of Calcein by living cells, therefore its use is limited to live cultures while the TC can also be used in fixed samples [3, 13]. In this regard, it is worth noting that TC allows for the multiplex staining with fluorophores visible in other channels, including the nuclear dye Hoechst 33258 and TRITC-conjugated Phalloidin as shown in our study, and is therefore a versatile tool for immunohistochemistry analysis. Moreover, our results shown that the quantitative assessment of mineralization at specific time points can be achieved by an incubation of less than 24 hours with TC resulting in quantifiable and visible signal.

In summary, in this study we have developed a new protocol for the quantitative monitoring and quantitation of cell mineralization in real time based on the use of Tetracycline Hydrochloride. In comparison to conventional histological methods such as Alizarin Red and Von Kossa staining, which are endpoint assays and require multiple steps for staining and dye quantification, the use of TC is cytocompatible, can be performed in live cells lowering the number of cells required for longitudinal studies, and is both straightforward and economical.

Overnight incubation results in visible and quantifiable fluorescence signal, which can be quantified by spectrophotometry directly from live cultures while being compatible with the use of other fluorophores in live and fixed samples, enabling multiplex immunohistochemistry analysis. TC cell labelling enables the *in vitro* evaluation of osteogenic differentiation and can thus support research into bone repair and tissue engineering targeting new pro-osteogenic approaches.

Conclusions

In this study we have described a new non-destructive method for the live and quantitative staining of mesenchymal stem cell cultures undergoing osteogenic differentiation, based on the transient administration of Tetracycline hydrochloride during the culture period. The results showed this method to be non-toxic, sensitive, and quantitative. TC can be selectively imaged by fluorescent microscopy in live cells, and enables the concomitant use of other fluorophores, which offers a useful and versatile method for high throughput analysis of osteogenic cultures.

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Author Disclosure Statement

The authors declare that they have no conflict of interests.

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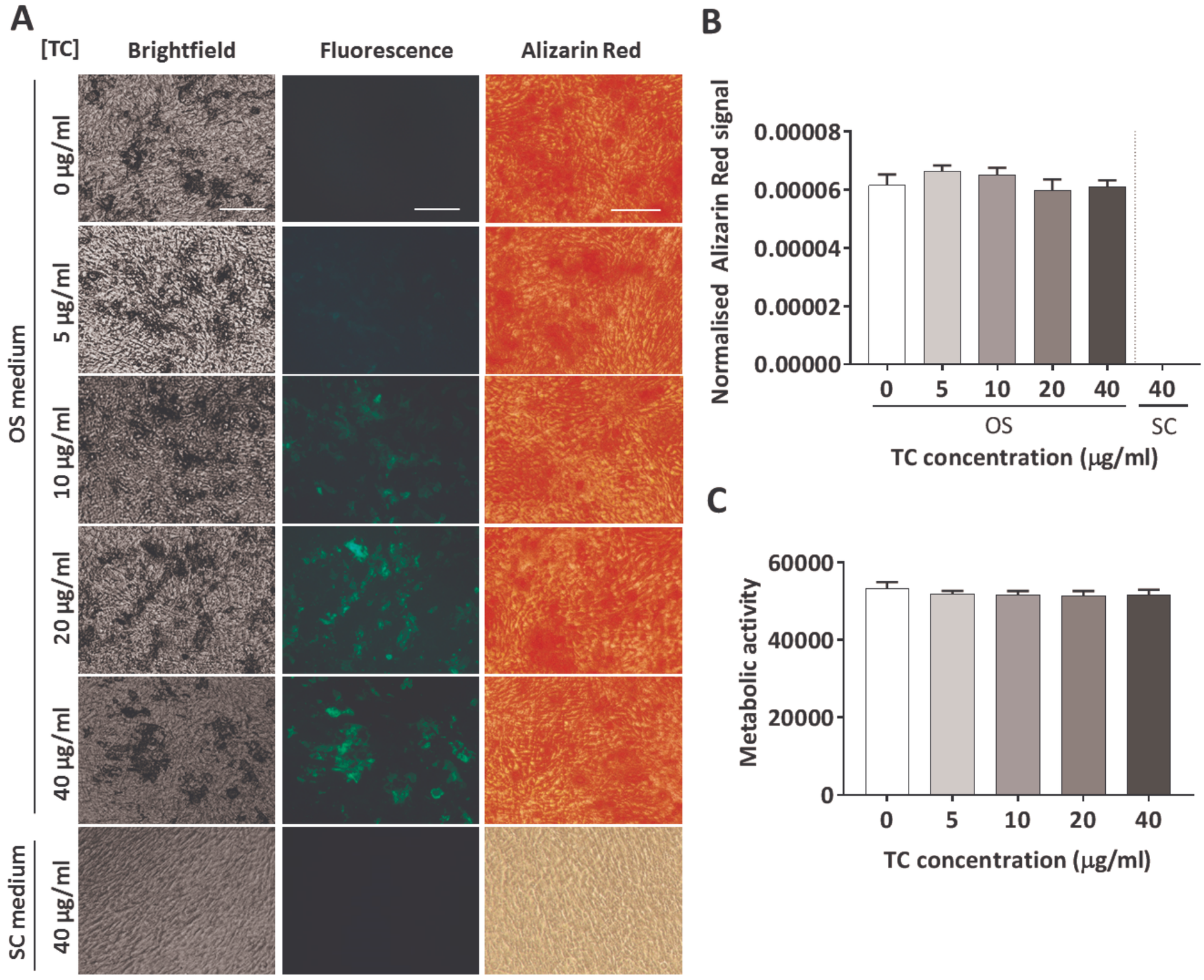
A
[TC]
Brightfield
Fluorescence
Alizarin Red
OS medium
0 µg/ml
5 µg/ml
10 µg/ml
20 µg/ml
40 µg/ml
SC medium
40 µg/ml
B
Normalised Alizarin Red signal
0.00008
0.00006
0.00004
0.00002
0.00000
0
5
10
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40
40
OS
SC
TC concentration (µg/ml)
C
Metabolic activity
60000
40000
20000
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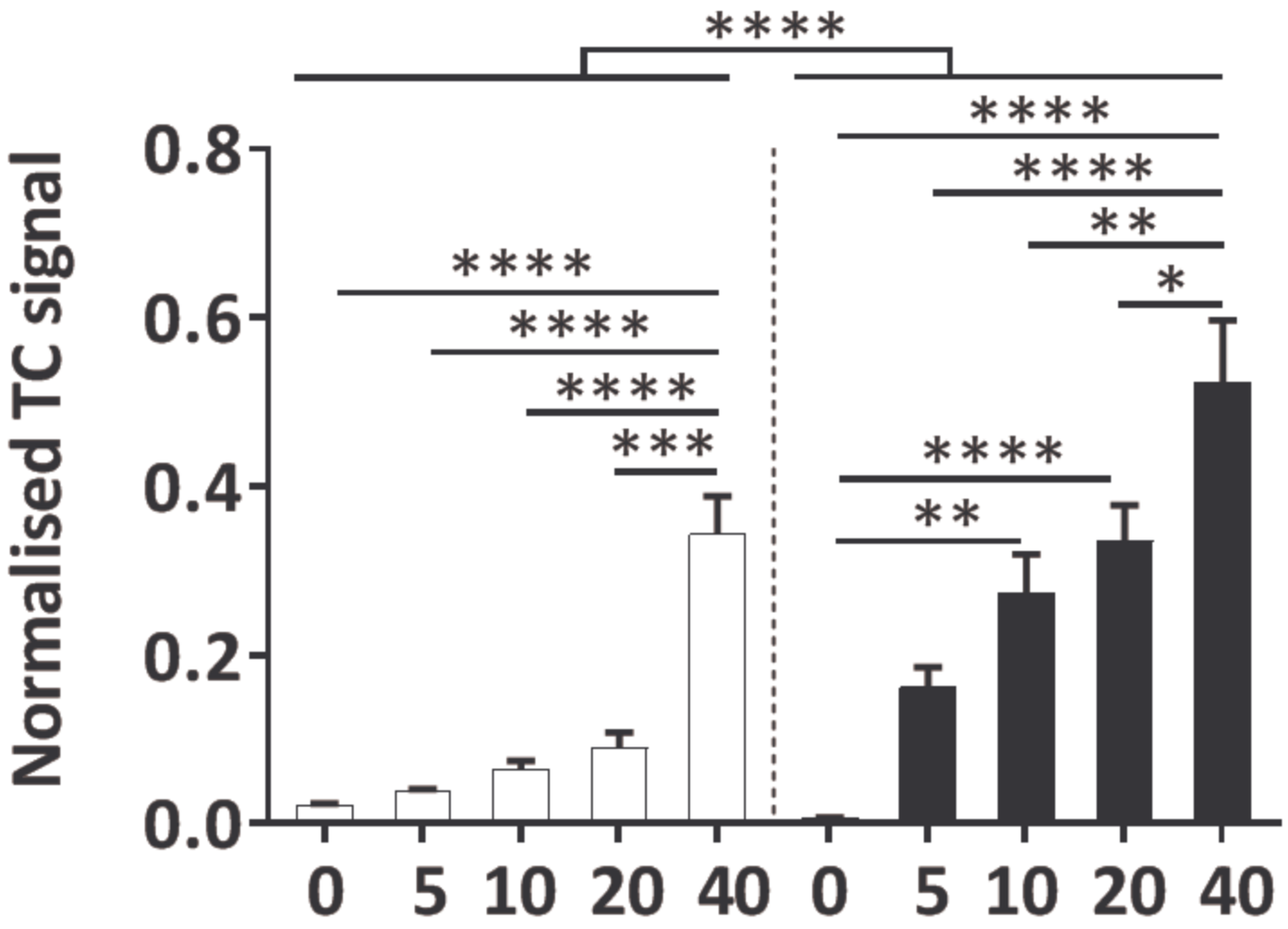
5
10
20
40
TC concentration (µg/ml)

Fig. 1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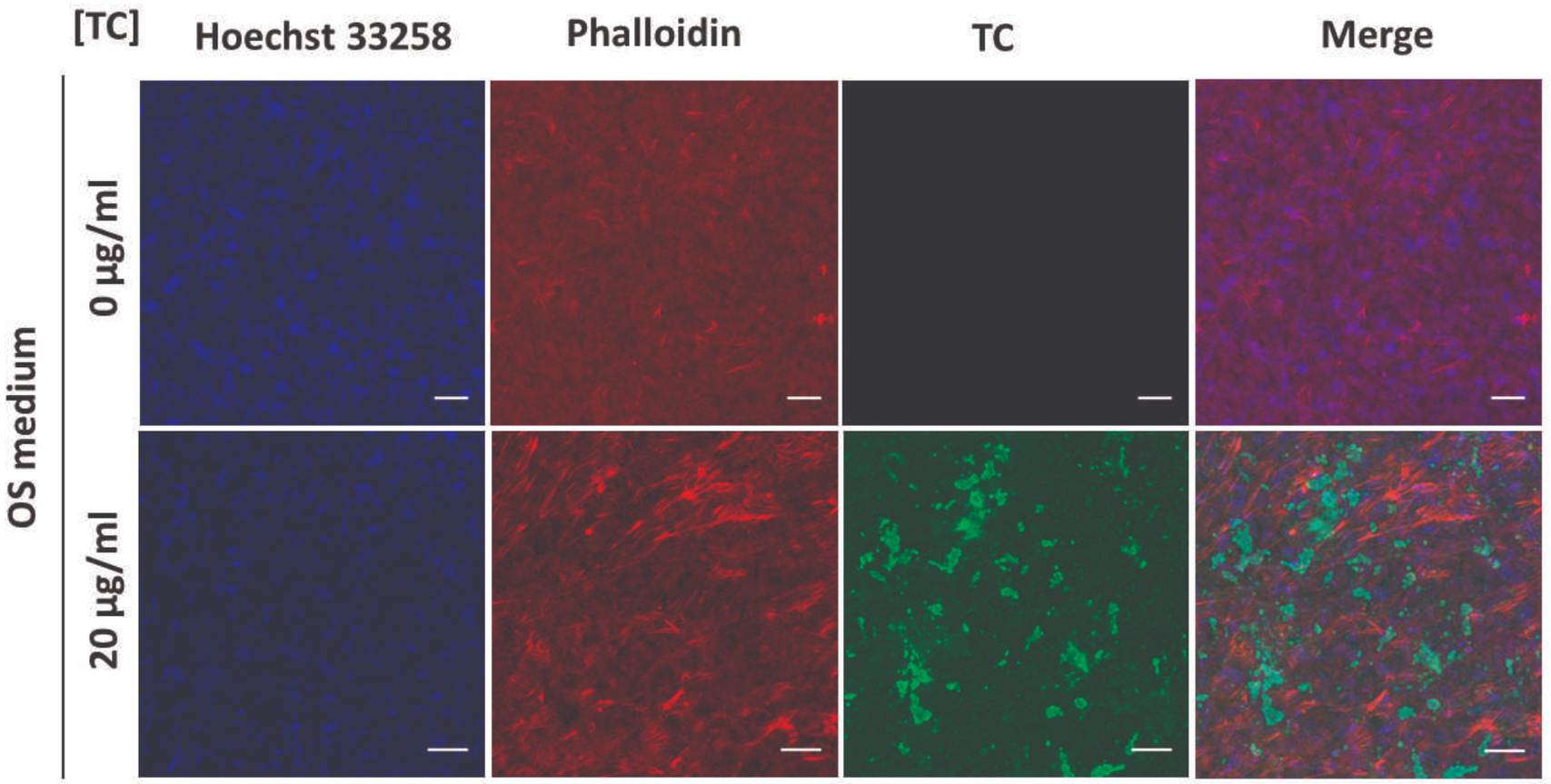
Normalised TC signal
0.8
0.6
0.4
0.2
0.0
0
5
10
20
40
0
5
10
20
40
TC concentration (μg/ml)

Fig. 2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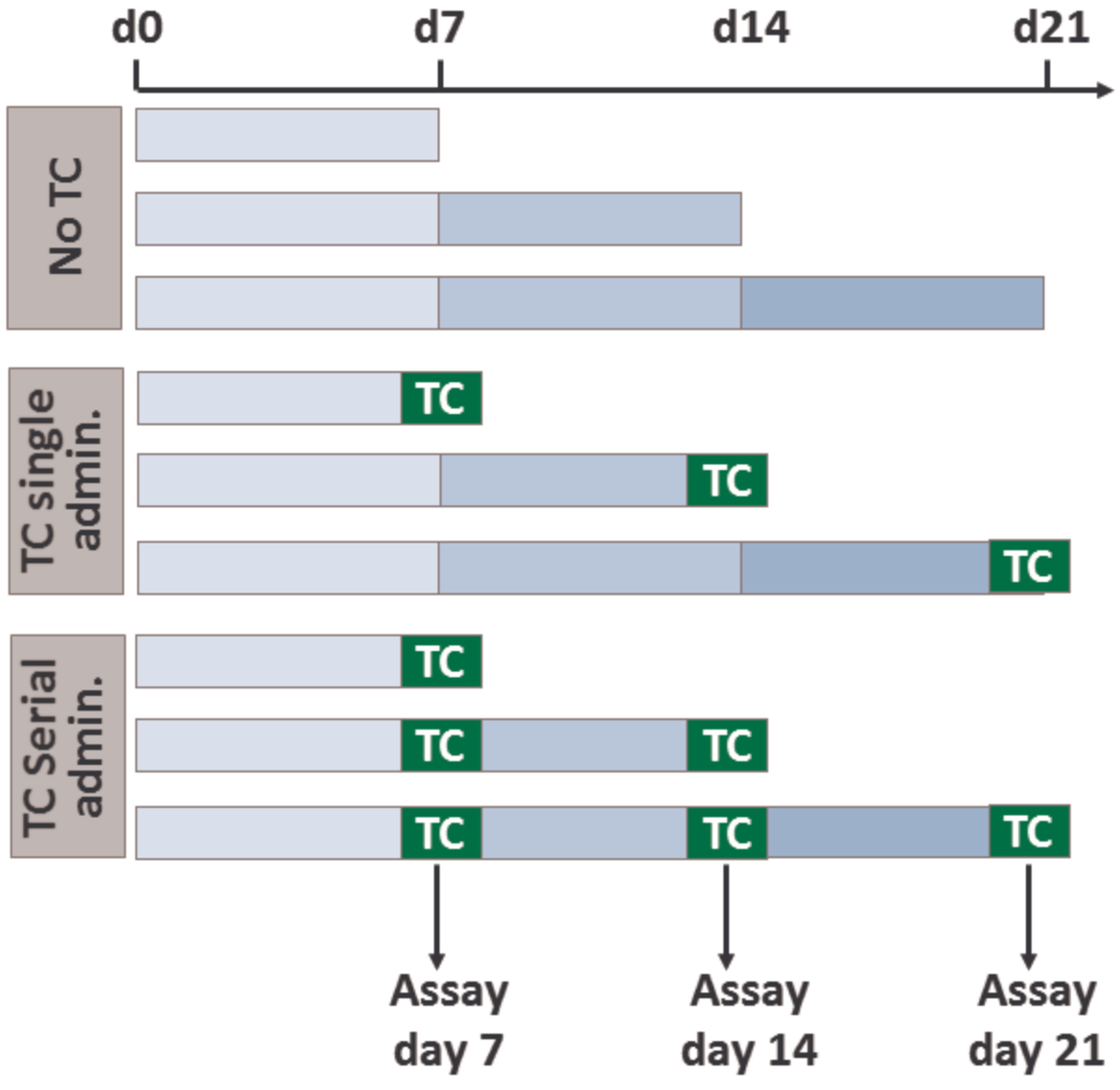
Hoechst 33258
Phalloidin
TC
Merge
OS medium
0 μg/ml
20 μg/ml

Fig. 3

A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d7
d14
d21
No TC
TC single admin.
TC Serial admin.
TC
Assay day 7
Assay day 14
Assay day 21

B

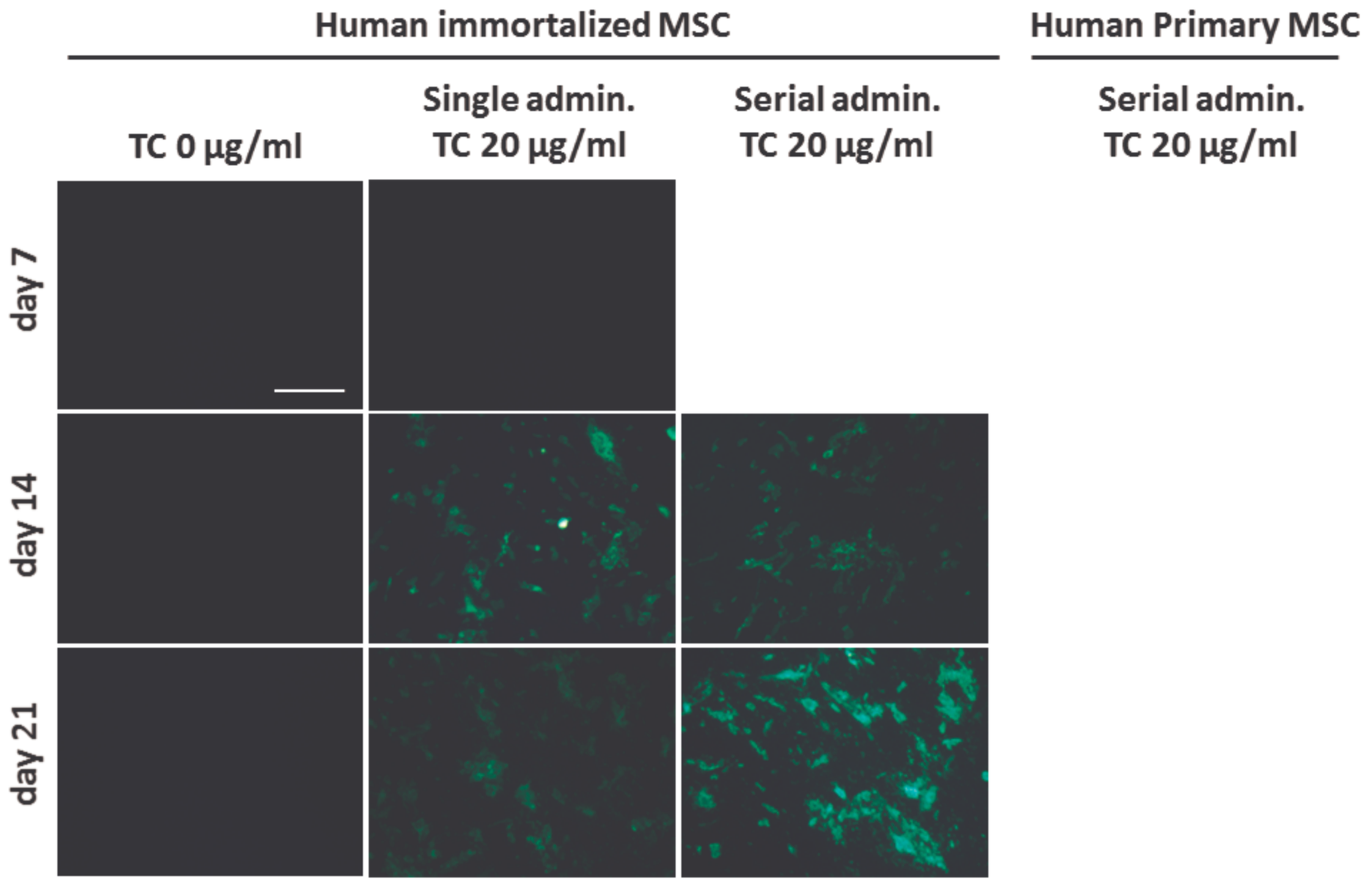

Human immortalized MSC
Human Primary MSC
TC 0 µg/ml
Single admin. TC 20 µg/ml
Serial admin. TC 20 µg/ml
Serial admin. TC 20 µg/ml
day 7
day 14
day 21

Fig. 4

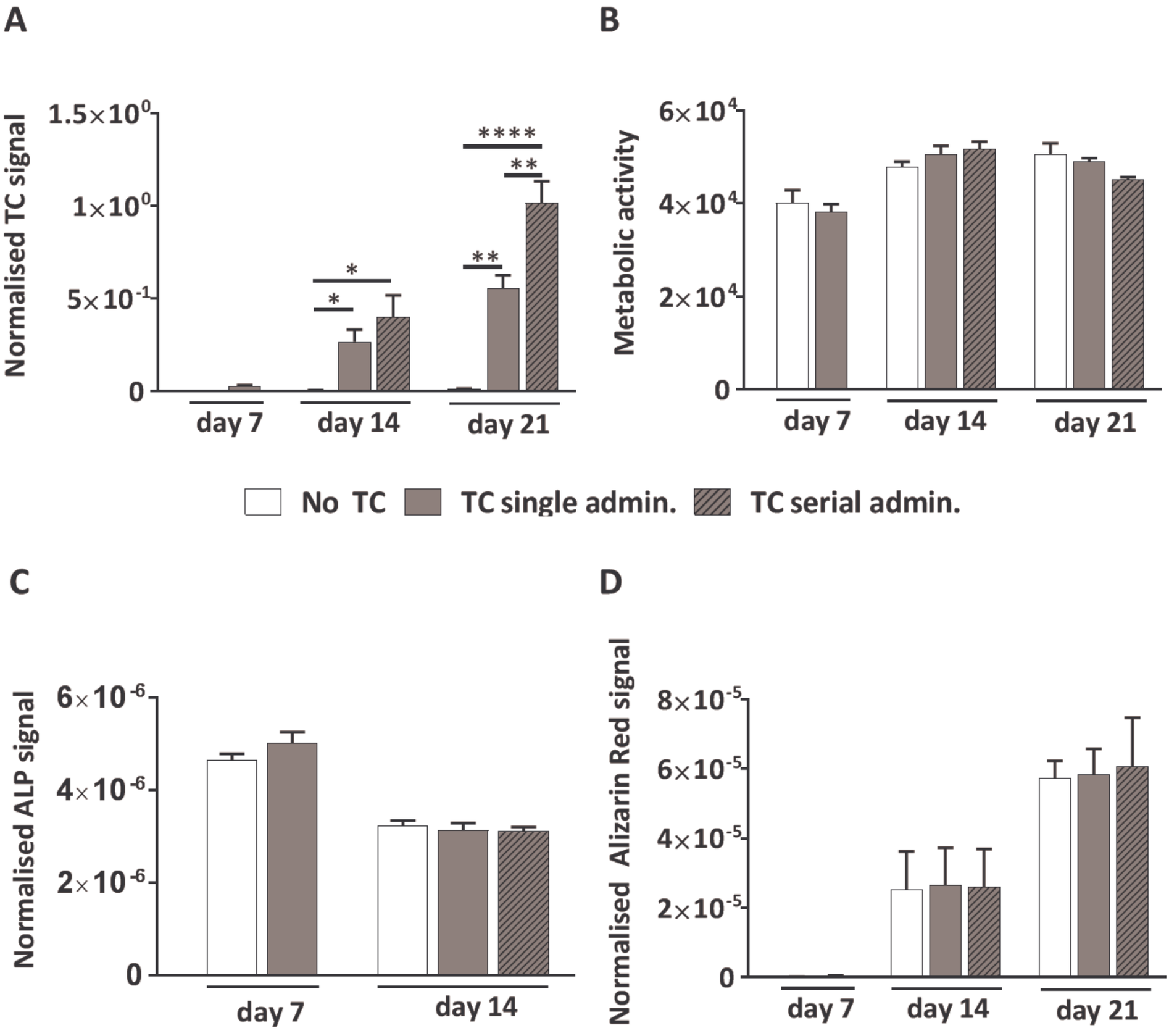
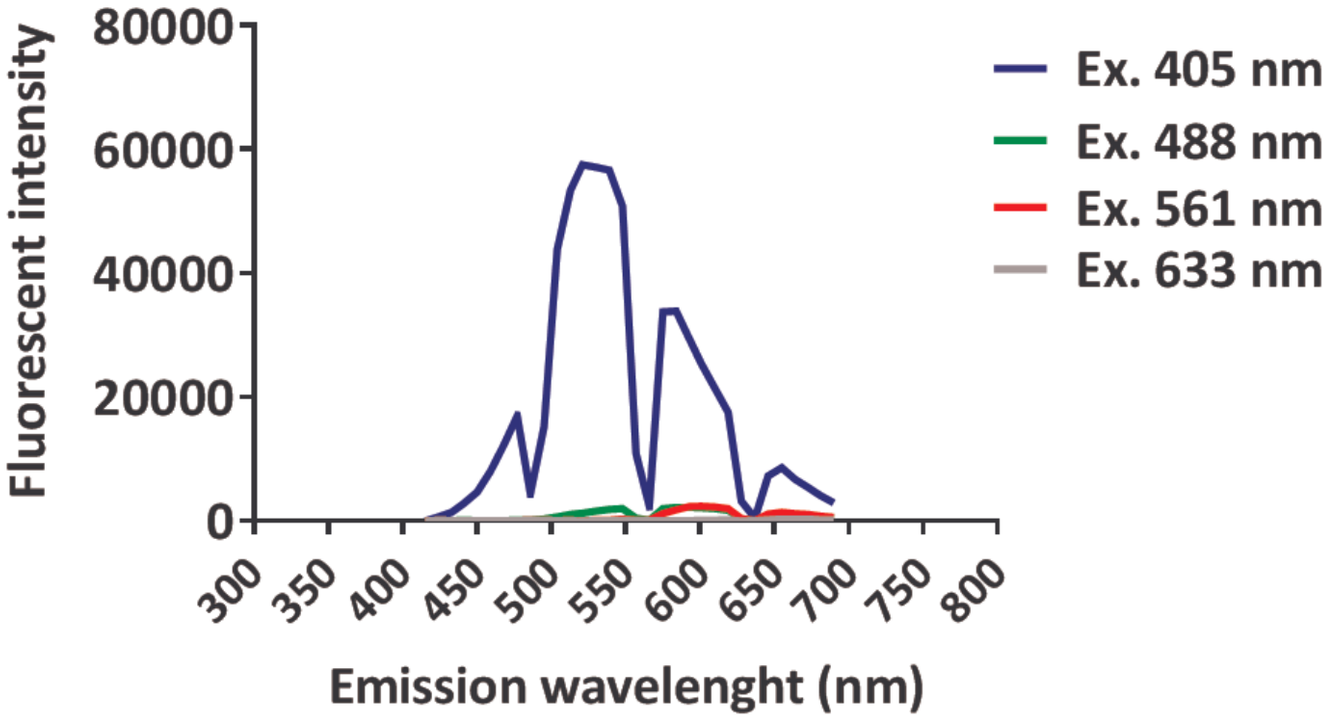
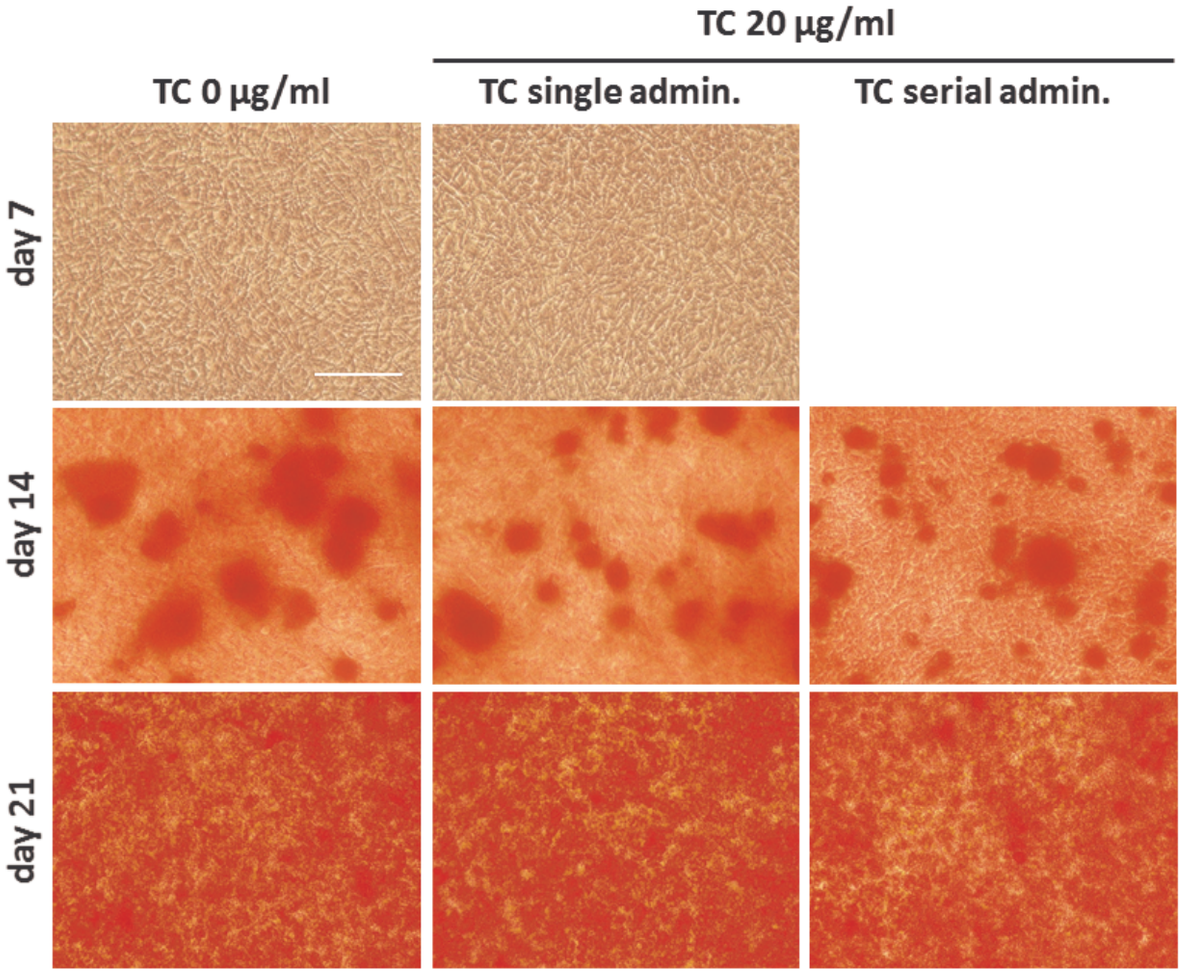

A
B
C
D
Normalised TC signal
1.5×10^0
1×10^0
5×10^-1
0
*
*
**
**
****
day 7
day 14
day 21
Metabolic activity
6×10^4
4×10^4
2×10^4
0
day 7
day 14
day 21
No TC
TC single admin.
TC serial admin.
Normalised ALP signal
6×10^-6
4×10^-6
2×10^-6
0
day 7
day 14
Normalised Alizarin Red signal
8×10^-5
6×10^-5
4×10^-5
2×10^-5
0
day 7
day 14
day 21

Fig. 5


Fluorescent intensity
80000
60000
40000
20000
0
300
350
400
450
500
550
600
650
700
750
800
Emission wavelenght (nm)
Ex. 405 nm
Ex. 488 nm
Ex. 561 nm
Ex. 633 nm

Suppl. Fig. 1


TC 20 µg/ml
TC 0 µg/ml
TC single admin.
TC serial admin.
day 7
day 14
day 21

Suppl. Fig. 2