Introduction of Macarpine as a novel cell-permeant DNA dye for live cell imaging and flow cytometry sorting

Running title: Live cell imaging and sorting with Macarpine

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Abbreviations: FACS, Fluorescence-activated cell sorting; MA, macarpine; MEFs, mouse embryonic fibroblasts; PI, propidium iodide; QBA, quaternary benzophenanthridine alkaloid; VR, Vybrant$^{(R)}$DyeCycle^{TM} Ruby; VO, Vybrant$^{(R)}$DyeCycle^{TM} Orange

Abstract

Background Information: Macarpine is a quaternary benzophenanthridine alkaloid isolated from plants *Macleaya microcarpa* or *Stylophorum lasiocarpum*. Benzophenanthridine alkaloids are interesting natural products which display anti-proliferative, antimicrobial, antifungal and anti-inflammatory activities and also fluorescence properties. In a previous study, we demonstrated that macarpine thanks to the ability to interact with DNA and thanks to its spectral properties could be used as a supravital DNA probe for fluorescence microscopy and flow cytometry including possibility of cell cycle analysis. In this study macarpine was evaluated as DNA dye suitable for time-lapse microscopy and flow cytometric sorting.

Results: A375 cells stained with macarpine were monitored by time-lapse microscopy for 24 h. During the first 2-3 h mitoses were observed at macarpine concentrations up to 0.5 μg/ml. After this period cells at macarpine concentrations of 0.75 and 0.5 μg/ml underwent apoptosis. Cells cultivated with macarpine at concentration of 0.25 μg/ml or lower survived throughout the 24 h period. However at macarpine concentration of 0.25 μg/ml they stop mitosis during first 4 h. Toxicity of macarpine was dependent on light used and frequency of image capturing. Intensity of macarpine fluorescence decreased during the incubation. The macarpine concentration of 0.1 μg/ml was assessed as the best for live imaging with respect to fluorescence intensity and toxicity.

Macarpine at concentration 10 μg/ml was used for sorting of EGFP-labelled neurons and fibroblasts yielding profiles similar to those obtained with DRAQ5. Contrary to DRAQ5, macarpine-stained neurons and fibroblasts survived in culture, and the sorted cells lost the macarpine signal suggesting reversible binding of dye to the DNA.

Conclusion: The results proved the possibility to use macarpine for depiction of chromosomes and monitoring of mitosis by time-lapse microscopy. Nuclear fragmentation during apoptosis

was also detected. In addition, this DNA dye is a suitable probe for sorting of EGFP-labelled, living cells including neurons, which survive this procedure.

Introduction

Dyes that bind to DNA in living cells are commonly used for chromatin visualization in live cell imaging and flow cytometry. Time-lapse microscopy is used to observe the dynamics of cell behaviour upon various physiological or stress conditions in real time. Commonly used supravital dyes are DAPI (4'-6-diamidino-2-phenylindole) and the bisbenzimide dyes Hoechst (33258; 33342; 34580) (Shapiro, 2003). Since time-lapse microscopy with these dyes usually requires UV excitation, their use is complicated not only by dye toxicity, but also by toxicity of UV light. Search for dyes excitable by less toxic light of longer wavelength can circumvent the problem of UV toxicity. Such dyes were recently introduced, i.e. synthetic antraquinone DRAQ5 (the deep red fluorescing agent, Smith et al., 1999, 2000), with excitation maximum near 700 nm, and SYTO and Vybrant$^{(R)}$DyeCycle^{TM} Ruby (VR) and Orange (VO) nucleic acid cell permeant stains (Molecular Probes, Eugene, OR).

FACS is a laser-based technique that allows the enrichment of selective cell populations defined by a specific gating procedure in a flow cytometer (Shapiro, 2003). This procedure can be used for the isolation of living cells at specific cell cycle stages when appropriate supravital DNA dyes are used, provided DNA labelling is stoichiometric. Nevertheless, the isolation of viable cells through sorting is largely hampered by the cytotoxic effect of the DNA dye, which could synergize with the damage triggered to the cells by the sorting procedure itself. Therefore seeking for further probes is desirable.

MA (Fig. 1) is a quaternary benzophenanthridine alkaloid (QBA) isolated from plants *Macleaya microcarpa* or *Stylophorum lasiocarpum* (Pěnčíková et al., 2011, Šebrlová et al., 2015). QBAs are interesting natural products which display anti-proliferative, antimicrobial, antifungal and

anti-inflammatory activities (rewieved by Slaninová et al., 2014). They are coloured in the spectrum of yellow to dark red, and display fluorescence properties. Urbanová et al. (2009) characterized fluorescence excitation and emission spectra of selected QBAs, including MA, alone and in the presence of CT DNA. These authors concluded that QBAs could be excited by light of wavelength of 300-550 nm. Due to 18-fold increased fluorescence intensity of MA in excess of CT DNA, MA was found to be promising for use as a DNA probe. In this regard, a previous work from our laboratory suggested that MA could be used as a supravital DNA dye for fluorescence microscopy and flow cytometry, including the possibility of the cell cycle analysis (Slaninová et al., 2007).

This study was designed to assess the possible use of MA as a supravital DNA dye for time-lapse microscopy and flow cytometry sorting. We have proved the ability of MA to depict dynamics of mitosis and apoptosis by lifetime imaging and to be used for sorting of cells in specific cell cycle stages. We also demonstrated the possibility of double staining MA and EGFP.

Results

Microscopy of living cells

Using a fluorescence microscope, we have determined the concentrations of MA that allow the staining of living cells and their long-term survival. We have found that MA is able to stain nuclei starting at concentration of 0.01 μg/ml. However at the lowest concentrations its fluorescence quickly faded. Fluorescence of MA at concentration of 0.01 μg/ml disappeared during the first hour, while at a concentration of 0.05 μg/ml it persisted during 48 h. Background fluorescence was relatively high at these two lowest concentrations. Much stronger fluorescence of nuclei was observed using MA at concentration 0.1 μg/ml. This concentration appeared to be the most suitable for long-time observation because nuclei were still visible and

the cells were viable after 48 h. At higher concentrations (0.25 μg/ml and higher) signal of MA was stronger, but cells underwent apoptosis within 48 h (data not shown).

To assess the possibility of MA use in two-colour fluorescence, H2-B2T-EGFP neuroepithelial living cells were labelled with MA. These cells express EGFP at variable levels depending on the cell cycle stage they are (García-Dominguez et al., 2011). This analysis demonstrated that MA is able to stain cell nuclei even in those cells expressing EGFP at the highest levels (Fig. 2).

Time-lapse microscopy

Using a Confocal LSM 700 Laser Scanning Microscope we tested the possibility of MA application in time-lapse microscopy. A375 cells stained with MA at the concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 and 0.75 μg/ml were monitored by time-lapse microscopy for 24 h. The intensity of fluorescence increased with increasing MA concentration. Fluorescing vesicles near the nucleus were also visible. When recorded at 15 min. intervals cells survived at all tested concentrations. We demonstrated the ability of MA to visualise mitotic chromosomes. During the first 2-3 h mitosis was observed at all tested MA concentrations. When videos were recorded at 5 min. intervals cells at MA concentrations of 0.75 and 0.5 μg/ml underwent apoptosis after 4 h of monitoring. Cells cultured with MA at a concentration of 0.25 μg/ml and lower survived during the whole time-lapse monitoring period (24 h) and twenty-four additional hours in the cell incubator. However, 0.25 μg/ml MA prevented A375 cells to undergo mitosis during the first 4 h after treatment. Intensity of MA fluorescence decreased during the incubation suggesting release of MA from cells. Based on our initial results we set the concentration 0.1 μg/ml of MA as the best for live cell imaging with respect to fluorescence intensity and toxicity, including phototoxicity. Fig. 3 and Suppl video 1 depict the course of mitosis in cells stained

with MA at concentration 0.1 μg/ml. Video was recorded in 5 min. intervals. Fig. 4 and Suppl video 2 show apoptosis in cells stained with MA at concentration 0.75 μg/ml. Video was recorded in 15 min. intervals. The results proved that MA can be used for depiction of chromosomes and mitosis and also of nuclear fragmentation during apoptosis.

Cytometric analysis and sorting of MA-labelled EGFP$^+$ neurons

To test whether MA is able to stoichiometrically label nuclear DNA of non-proliferating living cells, we took advantage of the existence of a small population of tetraploid (4C) neurons in the retina and adult brain of vertebrates (Morillo et al., 2010, López-Sánchez et al., 2011, 2014), which in the cerebral cortex represent around 3 % of all neurons (López-Sánchez and Frade, 2013). To this aim, living telencephalic cells from E17.5 mouse embryos expressing EGFP under the control of the neuronal-specific *Mapt* promoter (Tau-EGFP mice) were labelled with 10 μg/ml MA. Then, they were subjected to flow cytometry to verify if this dye can discriminate the low represented population of 4C neurons from those with 2C DNA content, and if MA labelling allows cell sorting without affecting the viability of the neurons.

As a positive control for DNA labelling, E17.5 telencephalic cells were permeabilized with Triton X-100 and labelled with PI. This procedure allowed to discriminate the 4C population and resolved the aggregates present in the sample (Fig. 5A). As can be seen in Fig 5A, the resolution of the MA-labelled populations based on its DNA content is quite similar to that obtained with PI. This indicates that MA is an optimal dye able to discriminate non-proliferating cells in terms of DNA content as well as to resolve aggregates in a similar manner as PI. The capacity of MA to discriminate DNA content was also compared with other dyes known to label DNA in living cells, including DRAQ5, VR and VO. Interestingly, the profile obtained with MA in living cells was similar to that obtained with DRAQ5, whereas VO could hardly resolve the 4C population and labelling with VR could not resolve the 4C population (Fig. 5A).

Furthermore, both MA and DRAQ5, but not VR, can be efficiently used for the quantification of DNA content in the $EGFP^+$ cells (i.e. neurons) (Fig. 5B). This is not the case for VO since the emission signal of this dye overlaps with that of EGFP, thus precluding its use for the quantification of DNA content in $EGFP^+$ cells.

Despite the relatively high concentration of MA required for supravital labelling of neuronal nuclei, we observed that the sorting process resulted in the elimination of this dye from these cells. This conclusion was evidenced from experiments in which sorted $EGFP^+$ neurons with 2C DNA content were co-cultured with WT neurons (see Methods). These experiments demonstrated that the $EGFP^+$ neurons that survived the sorting procedure were able to adhere to the substrate and lose the MA signal within a few hours (Fig. 5C, left panels), suggesting that this compound has a reversible binding capacity to living neurons. In contrast, died neurons maintained the MA signal in the nucleus (data not shown). A substantial number of sorted $EGFP^+$ neurons survived for long periods of time (10 days *in vitro*) and developed neurites similar to those observed in WT neurons (Fig. 5C, right panels).

Cytometric analysis and sorting of MA-labelled $EGFP^+$ fibroblasts

To test whether MA can be useful for cell sorting experiments of $EGFP^+$ proliferating cells we decided to focus on mouse embryonic fibroblasts (MEFs) expressing this fluorescent protein under the control of a ubiquitous promoter (EGFP-MEFs). For this analysis, three different doses of MA were tested (10, 20 and 50 μg/ml). MA used at 20 and 50 μg/ml was able to resolve the G0/G1 and G2/M populations, although it was not as good as PI in Triton X-100-permeabilized EGFP-MEFs (Fig. 6A**).** In contrast, the lowest dose of MA used for this analysis (10 μg/ml), which could efficiently define the cell cycle in $EGFP^+$ neurons (see above), did not sufficiently resolved the different cell cycle phases in $EGFP^+$ MEFs (Fig. 6A). This observation

suggests that the concentration at which MA is used to label living cells in flow cytometric analyses needs to be adjusted for each cell type.

To study the toxicity of MA in these proliferative cells and its usefulness as DNA dye in sorting experiments, dissociated EGFP-MEFs were labelled with the three different doses of MA indicated above and those in the G2/M region were sorted and cultured. This procedure was able to enrich the percentage of EGFP-MEFs in G2/M, as evidenced by PI labelling of an aliquot of the sorted cells permeabilized with Triton X-100 (see example of 20 μg/ml MA treatment in Fig. 6B). We observed that around 70% of the EGFP-MEFs showed 4C DNA content, a proportion expected for an unrepresented population which in the original cell sample was below 15%. Our analysis indicated that, 24 h after plating, the toxicity of MA in sorted EGFP-MEFs clearly depends on the dose at which it is used (Fig. 6C). Virtually, all cells exposed to 10 μg/ml MA were able to survive and proliferate for several days after plating. These cells lacked MA staining in their nuclei (Fig. 6C upper panels). In contrast, some signs of toxicity appeared in EGFP-MEFs treated with 20 μg/ml of MA as some cells were either un-adhered or poorly adhered, showing a rounded morphology with condensed nuclei and soma and with weak nuclear MA labeling (Fig. 6C middle panels). Sorted EGFP-MEFs exposed to 50 μg/ml of MA were totally unviable 24h after sorting (Fig. 6C bottom panels).

We therefore conclude that, as for other supravital DNA dyes, the optimal dose of MA must be optimized for each cell type. Nevertheless, depending on the purpose of the flow cytometric assay, some level of toxicity should be acceptable if this results in optimal DNA labelling and improved resolution of the cellular populations.

Toxicity of MA

Since lifetime imaging showed toxicity of MA, we performed cell viability and cytotoxicity assays in A375 cells to discriminate between toxicity of MA itself and phototoxicity upon

fluorescent light. Anti-proliferative activity after 48 h treatment with MA was tested using a colorimetric MTT assay, which detects metabolically active cells (specifically, the activity of mitochondrial succinate dehydrogenase). Reduction in metabolic activity is proportional to the toxicity of the tested compound. MA at concentrations 0.01, 0.03 and 0.1 μg/ml did not affect cellular metabolic activity, while at concentration 0.3 μg/ml it was strongly affected (35% of control) and at concentration 1 μg/ml in fact no metabolic activity was detected (Fig. 7A left). We tested the toxicity of MA at concentrations which could be used for time-lapse microscopy. Given that the emission spectrum of MA is similar as that of PI, the use of conventional propidium iodide (PI) exclusion assay was complicated. Therefore we used Live/Dead green agent for this purpose. While MA at concentration of 0.25 μg/ml revealed 18.7% of dead cells after 48 h treatment, MA at the concentration of 0.1 μg/ml was much less toxic showing 7.3% of dead cells, which is comparable with untreated control (Fig. 7A right).

The results proved very low toxicity of MA at concentrations up to 0.1 μg/ml. Differences in the results of MTT assay of metabolic activity and Live/Dead green cytotoxicity assay suggest that the cells first arrest growth and metabolic activity.

Cell cycle analysis

With respect to above mentioned results and the results of movies demonstrating a slight decrease of mitotic activity in the presence of MA we decided to determine whether MA affects the cell cycle progression. The results of 24 h incubation of A375 cells with MA confirmed results of tests of viability and lifetime imaging. We did not detect any changes in the cell cycle distribution upon treatment with MA at concentrations of 0.05 and 0.1 μg/ml, while at MA concentration of 0.25 μg/ml percentage of cells at G1 was increased up to 76.8% (comparing

56% at control). Cell cycle distribution was completely changed at MA concentration of 0.5 μg/ml showing high percentage of necrotic and/or apoptotic cells at sug-G1 fraction (Fig. 7B).

Long time live cell imaging

To differentiate toxicity of MA itself and phototoxicity induced by fluorescence light we performed long-term observation (6 days) of MA treated A375 cells on BioStation using only phase contrast microscopy. Contrary to fluorescence imaging, phase contrast microscopy had no effect on cell division and viability of A375 cells treated with 0.1 μg/ml MA as these cells were alive and proliferated for all 6 days. Fig.8 shows cells at given time points – demonstrating normal division of cells upon treatment with MA at concentration 0.1 μg/ml (Fig.8A) and apoptosis after 200 min of treatment with MA at concentration 0.25 μg/ml (Fig.8B). By comparing these results with results of fluorescent microscope-based experiments testing the MA toxicity, it is apparent that MA at concentration of 0.1 μg/ml shows phototoxicity when combined with blue laser but not normal light, however MA concentration of 0.25 μg/ml is toxic even upon illumination with normal light.

Online Supplementary Material

Supplementary Video 1: Time-lapse fluorescence microscopy movie of A375 cell stained with MA at concentration 0.1 μg/ml obtained by Confocal LSM 700 Laser Scanning Microscopy using Plan-Apochromat ×63, 1.4 Oil DIC M27 objective (Zeiss) and 488 nm laser (3%) and detected at 518 nm (wavelength Alexa Fluor 594 channel, red) and bright field channel. The movie was recorded at 5 min intervals. The movie has a speed of 1 frame per second. Bar: 5 μm.

Supplementary Video 2: Time-lapse fluorescence microscopy movie of A375 cell stained with MA at concentration 0.75 μg/ml obtained by Confocal LSM 700 Laser Scanning Microscopy

using Plan-Apochromat ×63, 1.4 Oil DIC M27 objective (Zeiss), using 488 nm laser (0,8%) and detected at 518 nm (wavelength Alexa Fluor 594 channel, red) and bright field channel. The movie was recorded at 15 min intervals. The movie has a speed of 5 frames per second. Bar: 5 μm.

Discussion

In this study we have established conditions for application of MA as a DNA probe suitable for time-lapse imaging and flow cytometry cell sorting based on DNA content.

In our previous study we demonstrated the ability of MA to stain DNA and the possibility to use it as a supravital nucleic acid dye for both fluorescence microscopy and flow cytometry (Slaninová et al., 2007). In this regard, MA has several advantages. Since MA is excitable with common argon laser (488 nm) and binds DNA in a stoichiometric manner it could be used for quantitative studies including cell cycle analysis on common flow cytometers (Slaninová et al., 2007). Cell cycle analysis is widely used in the study of cell growth and defects in cell cycle regulation, oncology research and determinations of ploidy. Hoechst 33342 was mostly used for nuclei visualisation and determination of DNA content in living cells (Shapiro, 2003, Errington et al., 2005). Even though Hoechst 33342 itself is relatively non-toxic, its use in the lifetime imaging is not ideal because of requirement of UV excitation. This is disadvantageous for long-term imaging due to UV toxicity combined with phototoxicity of dye and for flow cytometry applications because of necessity of the UV laser. Flow cytometers equipped with UV lasers are not common because of the cost of both the laser and optics. Despite the new DNA probes excitable by blue laser such as DRAQ5 (Smith et al., 1999, 2000, Errington et al., 2005), and Vybrant$^{(R)}$DyeCycle^{TM} Green, Orange, and Ruby Stains have been recently introduced for cell cycle analysis, identification of additional cell permeant low toxic fluorescent probes is useful.

In this study we evaluated the possibility of MA application in cell sorting according to ploidy. This procedure allows the isolation of cells in specific cell cycle stages, thus constituting a simple method for cell cycle synchronization. MA enabled sorting of EGFP-MEFs with 4C DNA content, even though the 4C population in the samples represented only about 15% of total cells. The sorted EGFP-MEFS were highly enriched and, not only survived the MA treatment and sorting, but were also able to proliferate and grow for several days. Therefore, we believe that MA is a good candidate for cell cycle synchronization of proliferating cells through FACS. The release of the dye during the sorting process likely facilitates cell survival and proliferation. This view is consistent with the observation that persistent application of MA at lower doses than those used for sorting led to accumulation of cells at the G1 phase and cell death. This is also in agreement with the observation that other cell-permeant dyes that bind DNA with high strength prevent G2/M transition in the treated cells, which eventually leads to apoptosis (Martin et al., 2005).

Cell sorting of neurons was focused on the 2C population since the proportion of 4C neurons in our samples was too low to obtain a significant enrichment after a single round of sorting. Sorting of diploid neurons using MA was successful, and despite the high concentration of MA necessary for cell cycle analysis MA was non-toxic and enabled the culture of the sorted neurons for several days. DRAQ5 is also able to label and discriminate the 4C neuronal population in a similar way to MA, but contrary to the latter this dye is cytotoxic (Smith and Errington, 2006), and has been shown to lead to DNA damage response (Zhao et al., 2009). As in the case of EGFP-MEFs, low toxicity of MA in sorted neurons could be justified by our finding that after washing MA disappeared from the cells. Also our time-lapse experiments show fading of MA, suggesting its releasing from cells could facilitate cell survival.

In time-lapse microscopy the use of DNA probes is limited not only by toxicity of the probe, but also by phototoxicity. It was described previously that repetitive imaging of cells containing

DNA probe is toxic (Purschke et al., 2010, Ge et al., 2013). Recent studies show that not only UV, but also visible and blue light cause DNA damage probably due to the production of ROS (Ge et al., 2013). In addition to phototoxicity it is also necessary to take into account the fact that the DNA probes intercalate or otherwise interact with DNA and these interactions may influence the morphology and functions of chromosomes, which could lead to DNA damage (Zhao et al., 2009, Wojcik and Dobrucki, 2008). Deleterious effect of Hoechst to DNA was described by several authors (Durand and Olive, 1982, Singh et al., 2004). Also DRAQ5 caused chromatin condensation and changes at histones H1 and H2B distribution (Wojcik and Dobrucki, 2008). The most moderate effect on DNA and chromatin was observed upon treatment with minor groove binder SYTO 17 (Wojcik and Dobrucki, 2008, Zhao et al., 2009). Drawing all of our observations together, it seems clear that MA alone is low toxic up to concentration 0.3 μg/ml. MA at concentration of 0.1 μg/ml is non-toxic and thanks to intensity of fluorescence suitable for lifetime imaging. However our experiments proved the fact that use of MA is similarly as at the case of other supravital DNA dyes limited by phototoxicity. Toxicity of MA was dependent on frequency of imaging. Using blue laser in confocal time-lapse microscopy recorded at 5-15 min intervals we observed mitosis only during first 4-5 h, after this time period, cell division stopped, but cells survive. Differences in the results of MTT (metabolic activity assay) and Live/Dead green agent (viability assay) also demonstrate that at the first step cellular growth is arrested. This result was confirmed by cell cycle analysis showing accumulation of cells at G1 phase upon MA concentration of 0.25 μg/ml. At higher concentration (0.5 μg/ml) distribution of cell cycle was disrupted and significant sub-G fraction representing apoptotic and dead cells appeared. Time-lapse microscopy experiments on BioStation using only phase contrast imaging showed normal mitosis during period of several days at MA at concentration of 0.1 μg/ml, which proved non-toxicity of this concentration in contrast to its phototoxicity upon blue laser light. Contrary MA at concentration 0.25 μg/ml was

toxic even upon normal light illumination. These results demonstrate that time–lapse imaging is accompanied by phototoxicity which is stronger upon fluorescence light.

Here, we show that sorting and microscopy have different requirements on the concentration of MA, but also on the period of its action. While microscopy where MA is present all time is limited by phototoxicity, despite the concentration of MA is 100 times lower, sorting conditions enable long-term survival of sorted cells probably thanks to short-term action of MA. The concentration at which MA should be used depends on each cell type. The reason for this differential effect remains unclear, but it may depend on the specific metabolism of each cell type and/or whether the cell proliferates or is quiescent.

In conclusion we introduced in this paper MA as the cell-permeant nucleic acid dye compatible with important live cell applications. Firstly, MA can be used in short term time–lapse imaging experiments enabling monitoring of course of mitosis and apoptosis. Secondly, MA is appropriate tool for sorting of living cells based on DNA content, allowing cell survival and post-sorting cultivation. Thirdly, MA allows the simultaneous co-staining of the cells for other parameters and combining cell cycle analysis with additional live cell applications (i.e., analysis of EGFP). Finally, we would like to point out that MA thanks to the temporary binding to DNA is less toxic than DRAQ5 and VR. In light of these issues, we anticipate broad range of MA applications in sorting of living cells and in live cell imaging.

Material and methods

Macarpine

Macarpine (MA) (fig. 1) used in our experiments as a form of MA chloride was obtained from *Stylophorum lasiocarpum* as previously described by Šebrlová et al. (2015). Briefly, dried ground roots were extracted by methanol. The extract was then concentrated and dissolved in sulfuric acid. After filtration, the extract was alkalized and MA and other QBAs were

extracted into diethyl ether. Mixture of alkaloids was separated by semipreparative HPLC. The identity of pure MA chloride was checked by LC-MS/MS spectrometry. The purity of MA chloride was 98 %.

Mice

Tau-EGFP ($Mapt^{tm1(EGFP)Klt}$/J) mice were obtained from The Jackson Laboratory. This mouse strain expresses a cytoplasmic form of EGFP protein fused with the first 31 aminoacids of Tau protein. In these mice EGFP signal can be detected in the central nervous system (CNS) already at E10.5, resembling the neuron-specific TUJ1 antibody pattern (Tucker et al., 2001), thus indicating that only mature neurons express EGFP. To obtain $EGFP^+$ and $EGFP^-$ littermates, Tau-EGFP mice were backcrossed for two generations to CD1 mice, and the resulting Tau-$EGFP^+$ and wild-type (WT) embryos (E17.5) were used for this study. Embryos were staged as described by Kaufman (1992).

TgN(beta-act-EGFP)04Obs transgenic mice express EGFP protein under the control of cytomegalovirus enhancer and chick β-actin promoter, resulting in EGFP signal in all tissues except erythrocytes and hair (Okabe et al., 1997).

Experimental procedures were approved by the CSIC bioethics committee, in accordance with the European Union guidelines.

Cell lines and culture conditions

Human malignant melanoma cell line A-375 and EGFP-expressing H2-b2T immortalized neuroepithelial cells (García-Domínguez et al., 2011) were used in the experiments.

The A-375 cell line was purchased from the European Collection of Animal Culture (ECACC, Salisbury, U.K). Cells were grown in RPMI medium. The growth medium was supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml

streptomycin. The cultures were incubated at 37 °C under 5% CO_2 in a high-humidity-atmosphere and subcultured three times a week. H2-b2T immortalized neuroepithelial cells (Nardelli et al., 2003), engineered to stably express EGFP under the control of the cytomegalovirus promoter (H2-b2T-EGFP) (García-Domínguez et al., 2011), were maintained at 37ºC in RPMI 1640 medium containing GlutaMAX I, 25mM HEPES (Invitrogen), 10% fetal calf serum (FCS) (Invitrogen), and penicillin/streptomycin (P/S) (Invitrogen). As previously described, H2-b2T cell line is composed by a mixed population of tetraploid and mainly octoploid cells (García-Domínguez et al., 2011). In some instances, H2-b2T-EGFP immortalized neuroepithelial cells were cultured on coverslips (Menzel-Gläser) coated with poly-l-lysine (Sigma-Aldrich). G418 sulphate (1.5 mg/ml) was occasionally added to H2-b2T-EGFP neuroepithelial cells for the maintenance of EGFP expression in these cells.

Primary cultures

Cultures of mouse embryo fibroblasts stably expressing EGFP (EGFP-MEFs) were established from E13.5 TN(beta-act-EGFP)04Obs mouse embryos. Briefly, embryos were decapitated, eviscerated and minced in PBS, and then incubated for 30 min at 37 ºC with 1X Tripsin-EDTA solution (GIBCO). Reaction was stopped by adding 3 volumes of DMEM plus 10% of FCS and P/S (DMEM-FCS-P/S) and cells were then disaggregated by gently pipetting to obtain a single cell suspension. Samples were centrifuged for 5 min at 300 g and pelleted cells resuspended in fresh DMEM-FCS-P/S for plating. Medium was replaced every 3 days and EGFP-MEFs were expanded when density reached 70-80% confluence.

Neuronal cultures were performed as follows. Telencephalic vesicles of WT or Tau-EGFP$^+$ E17.5 embryos, the latter identified as those showing green fluorescent signal under blue light excitation, were isolated in sterile PBS. Then, they were cut into small pieces and incubated for 18-20 min at 37ºC in 1 ml of PBS containing 3 mg/ml bovine serum albumin (BSA) and 0.025%

trypsin (Worthington). Reaction was stopped by adding 3 volumes of DMEM-FCS-P/S and cells were mechanically dissociated by gently pipetting. Cells were pelleted by centrifugation 5 min. at 300 g and resuspended in fresh DMEM-FCS-P/S. At this point neurons were plated (WT) as described below, or used for cytometric analysis (Tau-EGFP neurons).

Supporting neuronal cultures were established by plating the WT neurons at a density of 20,000 cells/cm^2 on coverslips previously coated with poly-L-ornithine (p-orn) (Sigma-Aldrich). When cells adhered to the plate, approximately 1 h post plating, medium was carefully replaced with Neurobasal-B7-P/S. Half volume of this medium was replaced for freshly one every 3-4 days.

Staining of Living Cells

Fluorescence Microscopy

Fluorescence of alkaloids was observed in fluorescence microscope The Leica DMI4000 B automated inverted research microscope or a Nikon Eclipse E80i microscope. MA was dissolved in water at a concentration 1 mg/ml (stock solutions) and stored at -20°C. The stock solution was diluted with deionised water to obtain staining solutions (1-100 μg/ml). For microscopic observation 1 μl of the staining solution was added to a microscope slide and coverslip with the adhered cells was put on the drop directly from cultivating medium without previous washing or could be washed in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L KH_2PO_4, 6.5 mmol/L Na_2HPO_4, pH 7.4). For labeling of living cells, H2b-2T-EGFP neuroepithelial cells growing on poly-l-lysine were treated with 1 μl of 1 μg/ml of MA as indicated above, and microscopically analyzed.

Time-lapse fluorescence microscopy

For lifetime imaging experiments A375 cells were cultivated at µ-Slide 8 Well, ibiTreat: (Ibidy ibidi GmbH, Planegg / Martinsried, Germany). Cells were seeded at concentration 10^5/ml at 300 µl of RPMI medium. MA at final concentrations 0.01, 0.05, 0.1, 0.25, 0.5 and 0.75 µg/ml was added and slide was transported to microscopic stage. Time-lapse fluorescence microscopy movies were obtained by Confocal LSM 700 Laser Scanning Microscopy (Zeiss) by Plan-Apochromat ×63, 1.4 Oil DIC M27 objective, using 488 nm laser and detected at 518 nm wavelength (Alexa Fluor 594 channel, red) and bright field channel. Cells were maintained in a microscope stage incubator at 37 °C in a humidified atmosphere of 5% CO_2. The movies were recorded at 5 min (Fig. 3 Suppl video 1) and at 15 min (fig.4 Suppl video 2) and intervals. Microscopy images and movies were processed and analysed using ZEN 2012, Fiji, Photoshop, Corel-Draw X4, and Corel-Photo-Paint X4 softwares. The movies have speeds of 1 (Suppl video 1) or 5 (Supll video 2) frames per second.

Cytotoxicity assays

MTT assay

MTT assays were performed on 96-well plates (Nunc A/S, Rockilde, Denmark) by method that we described previously (Hammerová et al., 2011). The MTT assay was performed 48 h after alkaloid addition and the optical density was measured at 570 nm using DTX 880 multimode detector (Beckman Coulter, Inc.). The results are expressed as percentage of viable MA treated cells related to untreated control. Each concentration of each compound was examined in four replicate wells. The experiments were independently repeated three times.

Cell viability assay

Viability of A-375 cells upon MA treatment was measured using The LIVE/DEAD® Fixable Green Dead Cell Stain Kit (Life Technologies). A375 cells were seeded at concentration of 10^4

cells/ml to 24-well plates (Orange Scientific, Belgium) in 0.5 ml medium/well. After 24h cells were treated for 48h with MA at concentrations (0.1, 0.25 and 0.5 μg/ml). Thereafter, cells were harvested and processed according to manufacturer recommendation. Briefly, 1μl of the reconstituted fluorescent reactive dye was added to1 ml of the cell suspension, and incubated at room temperature or on ice for 30 minutes at dark. After washing, cells were resuspended in 900 μl of PBS and the percentage of dead (stained) cells was detected using a Cytomics FC 500 flow cytometry system (Beckman Coulter, Inc., CA, USA) at channel FL1 (emission at 525 nm). 10,000 cells per sample were analysed.

Cell cycle analysis

Approximately 5×10^5 cells/ml (A375) were treated with MA at concentrations 0.5, 0.1, 0.25, 0.5 μg/ml and cultivated at 12- well plates (Nunc A/S, Rockilde, Denmark) under standard conditions for 24 h. After incubation the cells were processed for propidium iodide (PI) staining by method described previously (Slunská et al., 2010). Cell cycle profile was analysed on a Cytomics FC 500 flow cytometer using the FL3 channel (emission at 620 nm), 10 000 – 20 000 events were acquired. Data from each sample were were analysed for cell cycle phases using software Multicycle AV for Windows (Phoenix Flow system, San Diego, U.S.A.). DNA content analysis included the determination of the percentage of G1, G2/M, S-phase and sub-G1 (apoptotic and/or necrotic) fractions.

Long time live cell imaging

BioStation CT (Nikon Instruments Inc.) was used for long time observation (6 days) of MA treated cells using phase contrast microscopy. Cells were seeded to 24-well plates at density and upon conditions described above. Phase contrast objective 10X was used. MA at

concentrations 0.1 and 0.25 μg/ml was applied. The movies were recorded in phase contrast mode at 15 min (MA 0.25) and 25 min. (MA 0.1) intervals for 6 days.

Cell Sorting

For flow cytometry sorting, we used a FACSAria cytometer (BD Biosciences) equipped with a double argon (488 nm) and helium-neon laser (633 nm). The emission filters used were BP 530/30 (FITC) (for EGFP), BP 616/23 (PE-Texas Red) (for VO and PI), and BP 780/60 (APC-Cy7) (for DRAQ5 and VR), and the data were analyzed with FACSDiva software (BD Biosciences). For sorting of Tau-EGFP neurons, suspensions of telencephalic cells from Tau-EGFP+ E17.5 mouse embryos were prepared as described above in DMEM-FCS-P/S at a density of 0.5-1.5×10^6 cells/ml, and then filtered through a 30 μm nylon mesh. To avoid apoptotic cell death while cells were waiting to be sorted, neuronal samples were maintained on ice in the presence of the pan-caspase inhibitor Z-VAD-FMK (BD Biosciences) at a final concentration of 25 μM. For DNA labeling, DRAQ5 was added to the cell suspensions (0.5×10^6 cells/ml) at 10 μM and incubated for 30 min at 37 ºC in the dark. Vybrant$^{(R)}$DyeCycleTM Orange (VO) and Vybrant$^{(R)}$DyeCycleTM Ruby (VR) at 5 μM or MA at a final concentration of 10 μg/ml were added to cells suspensions containing 1.5×10^6 cells/ml and incubated for 30 min at 37ºC in the dark. To avoid long incubation periods with the DNA dyes during long-lasting experiments, treatments were carried out sequentially on aliquots so that cells were exposed to the dyes for approximately 1-1.5 h.

The gating procedure employed for detection and analysis of DNA content in Tau-EGFP$^+$ neurons resembled to that previously described for neuronal cell nuclei (López-Sánchez and Frade, 2013), regardless of the DNA dye used. This procedure ensured the correct discrimination of doublets.

For EGFP-MEFs sorting, early passage (3-4) of growing, non-confluent MEFs cultures were trypsinized and the isolated cells were then resuspended at 1.5-2×10^6 cells/ml in DMEM-FCS-P/S containing 25 μM of Z-VAD-FMK. Cells were stained with different doses of MA for 30 min (10 μg/ml at 4 ºC or 20-50 μg/ml at room temperature), and subsequently sorted. Sorted cells were collected in DMEM-FCS-P/S medium and plated. Once the cells were adhered to the substrate, medium was replaced by fresh medium and cultured as described above. In order to verify the purity of the sorted population, a small number of 4C EGFP-MEFs was permeabilized by adding 2 volumes of PBS containing 0.1% of Triton X-100 (Sigma-Aldrich) and DNA was labeled with propidium iodide (Sigma-Aldrich) at a final concentration of 25 μg/ml. The DNA content of these previously sorted cells was re-analysed by flow cytometry and the purity of interested population was estimated.

Statistical analyses

At least three independent experiments were performed for each of the experiments described in this study. All the quantitative results were analysed with Statistica 12 (StatSoft CR s.r.o.) using the Student´s t-test. Data are represented as means ± SEM.

Author contribution

I.S. designed the study and experiments, performed time lapse experiments and was primarily responsible for the final preparation of the paper before submission. N.L.S. designed and performed the experiments with MEFS, H2b2T and neural cells, and was responsible for preparation of the part of the paper concerning FACS and sorting. O.V. performed cytotoxicity experiments. K.Š. and E.T. were responsible for MA isolation. E.T. and J.M.F. critically revised the paper.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Legends to figures

Figure 1. Structure of macarpine.

Figure 2. Supravital labelling of EGFP-H2b2T cells with MA. Proliferating H2b2T neuroepithelial cells expressing variable levels of EGFP were labelled with MA. MA nuclear labelling is evident in all cells and no interference with EGFP signal is observed. Bar: 10 μm.

Figure 3. Serial images showing mitosis obtained from time-lapse fluorescence microscopy. A375 cell were stained with MA at concentration 0.1 μg/ml. Movie was obtained by Confocal LSM 700 Laser Scanning Microscopy using 488 nm laser and detected at 518 nm wavelength (Alexa Fluor 594 channel, red) and bright field channel. Video was recorded at 5 min intervals. These time-lapse images are available in Supplementary video S1. Bar: 5 μm.

Figure 4. Serial images showing apoptosis obtained from time-lapse fluorescence microscopy. A375 cell were stained with MA at concentration 0.75 μg/ml. Movie was obtained by Confocal LSM 700 Laser Scanning Microscopy using 488 nm laser and detected at 518 nm wawelenght (Alexa Fluor 594 channel, red) and bright field channel. Video was recorded at 15 min intervals. These time-lapse images are available in Supplementary video S2. Bar: 5 μm.

Figure 5. MA labelling and sorting of EGFP-positive neurons. E17.5 telencephalic cells from Tau-EGFP mouse embryos were stained with PI (after permeabilization with Triton X-100) or *in vivo* with either MA or other cell-permeant DNA dyes (DRAQ5, VR, or VO), and subjected to flow cytometry (A,B). Then, EGFP-positive cells (i.e. neurons) with 2C DNA content were sorted and cultured in vitro (C). A. DNA content assessed by plotting height (-H) vs. area (-A) intensity levels for the indicated DNA Dyes. As expected, PI labeling is able to resolve doublets from singlets (diagonal box), as well as the diploid (2C) and tetraploid (4C) populations. Supravital DNA labeling with MA discriminates all the populations in a similar way as PI and DRAQ5, and better than VO and VR. B. DNA content, measured with the indicated DNA dyes

and plotted against EGFP signal intensity (FITC-A), allows to distinguish 2C and 4C EGFP-positive neurons as those located above the threshold line ($EGFP^+$). C. Diploid MA-labeled neurons (TAU-EGFP), isolated by FACS and co-cultured with non-labeled neurons for either 2 h or 10 days in vitro (10DIV) are shown. Nuclei were labeled with DAPI. Ph: phase contrast. Bar: 40 μm.

Figure 6. MA labelling and sorting of EGFP-positive MEFs. A. living EGFP-MEFs were stained with either PI (after permeabilization with Triton X-100) or MA, and subjected to flow cytometry. DNA content was assessed by plotting width (-W) vs. area (-A) intensity levels for the indicated DNA dyes used at the referred concentrations. Rectangles indicate singlets corresponding to cells in G0/G1 or in G2. B. The purity of the sorted EGFP-MEFs in G2 after treatment with 20 μg/ml MA, performed through PI labeling after permeabilization of the sorted cells with Triton X-100, reached 70%. C. EGFP-MEFs labeled with the indicated concentrations of MA, and then sorted in G2 and cultured for 24 h *in vitro* are shown. Some signs of toxicity appeared in EGFP-MEFs treated with 20 μg/ml of MA whereas 50 μg/ml MA resulted in non-viable cultures. Ph: phase contrast. Bar: 40 μm.

Figure 7. Cytotoxicity of MA. Graphs shoving the results of MTT assay (A left) and Live/Dead green agent assay (A right) and results of cell cycle analysis of PI stained A375 cells incubated with MA for 24 h (B). MA is non-toxic up to concentration of 0.1 μg/ml (A). *$p<0.0X$, Student's *t* test. The percentages of G1, S, G2/M and sub-G1-phase fractions are depicted. We had not detect any changes in cell cycle distribution upon treatment with MA at concentrations 0.05 and 0.1 μg/ml, while at MA concentration 0.25 μg/ml percentage of cells at G1 was increased and cell cycle distribution was completely changed at MA concentration 0.5 μg/ml showing high percentage of necrotic and/or apoptotic cells at sug-G1 fraction (B).

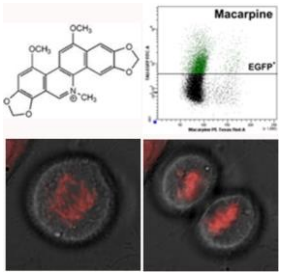
Figure 8. Serial images showing cells upon MA treatment obtained from BioStation time-lapse microscopy. A375 cell were treated with MA at concentration 0.1 and 0.25 μg/ml. BioStation CT (Nikon Instruments Inc.) was used for long time observation. The movies were recorded in phase contrast mode at 15 min (MA 0,25) and 25 min. (MA 0,1) intervals for 6 days. Normal division of cells upon treatment with MA at concentration 0.1 μg/ml (A) and apoptosis after 200 min of treatment with MA at concentration 0.25 μg/ml (B) is apparent. Bar: 20 μm.

Supplementary Video 1

Time-lapse fluorescence microscopy movie of A375 cell stained with MA at concentration 0.1 μg/ml obtained by Confocal LSM 700 Laser Scanning Microscopy using Plan-Apochromat ×63, 1.4 Oil DIC M27 objective (Zeiss) and 488 nm laser (3%) and detected at 518 nm (wavelength Alexa Fluor 594 channel, red) and bright field channel. The movie was recorded at 5 min intervals. The movie has a speed of 1 frame per second. Bar: 5 μm.

Supplementary Video 2

Time-lapse fluorescence microscopy movie of A375 cell stained with MA at concentration 0.75 μg/ml obtained by Confocal LSM 700 Laser Scanning Microscopy using Plan-Apochromat ×63, 1.4 Oil DIC M27 objective (Zeiss), using 488 nm laser (0,8%) and detected at 518 nm (wavelength Alexa Fluor 594 channel, red) and bright field channel. The movie was recorded at 15 min intervals. The movie has a speed of 5 frames per second. Bar: 5 μm.



Graphical abstract. In this report we show the possibility to use macarpine for monitoring of mitosis and apoptosis by time-lapse microscopy and for sorting of living cells. Macarpine concentration of 0.1 μg/ml was the best for lifetime imaging with respect to fluorescence intensity and toxicity. Macarpine at concentrations 10 and 20 μg/ml was used for sorting of EGFP-labelled cells based on its DNA content yielding profiles similar to those obtained with DRAQ5. Contrary to DRAQ5 MA stained cells survive. Cells growing in vitro lost the MA signal suggesting reversible binding of MA.

Figure 1.

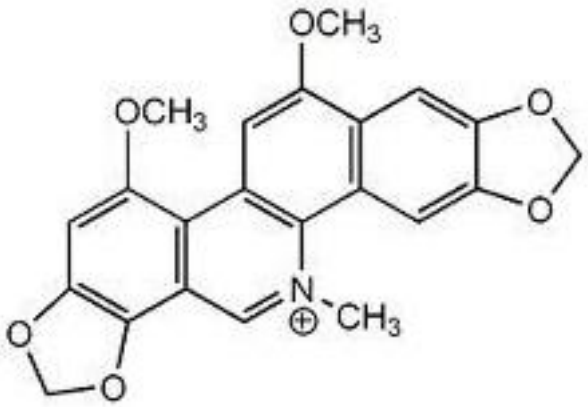
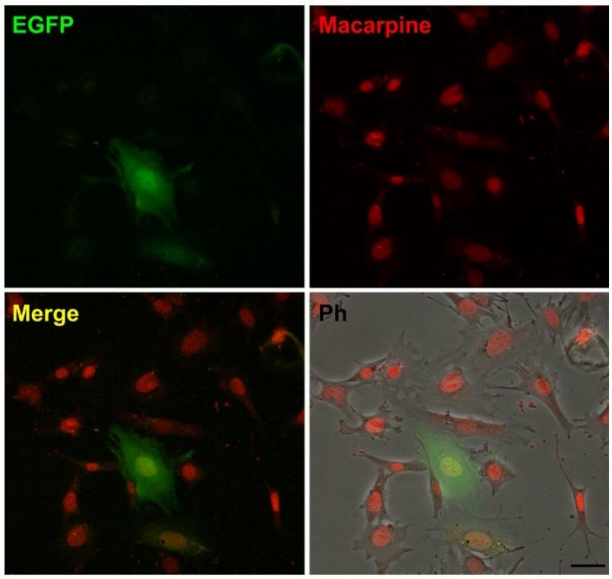


Figure 2.



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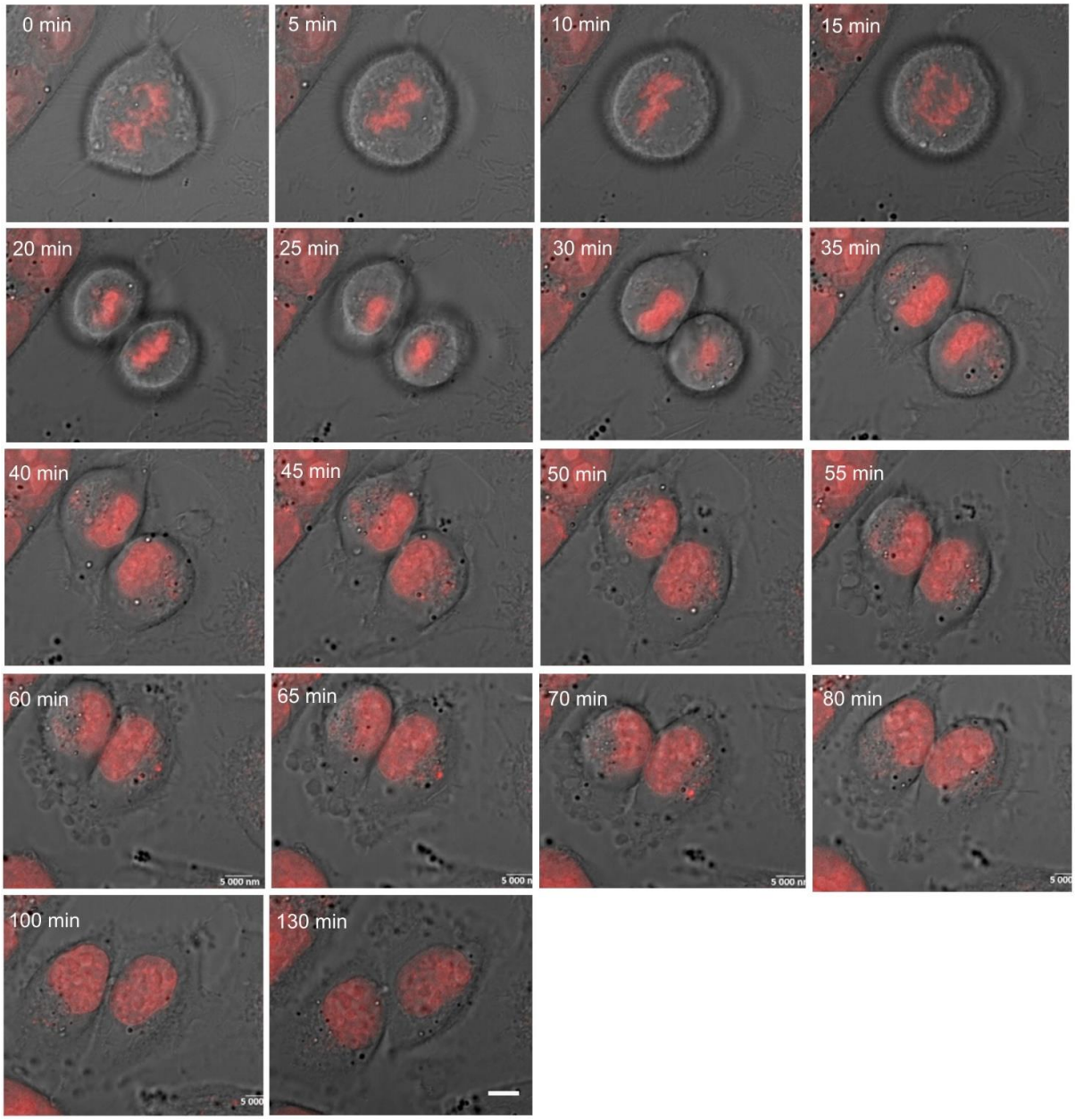
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Figure 4.

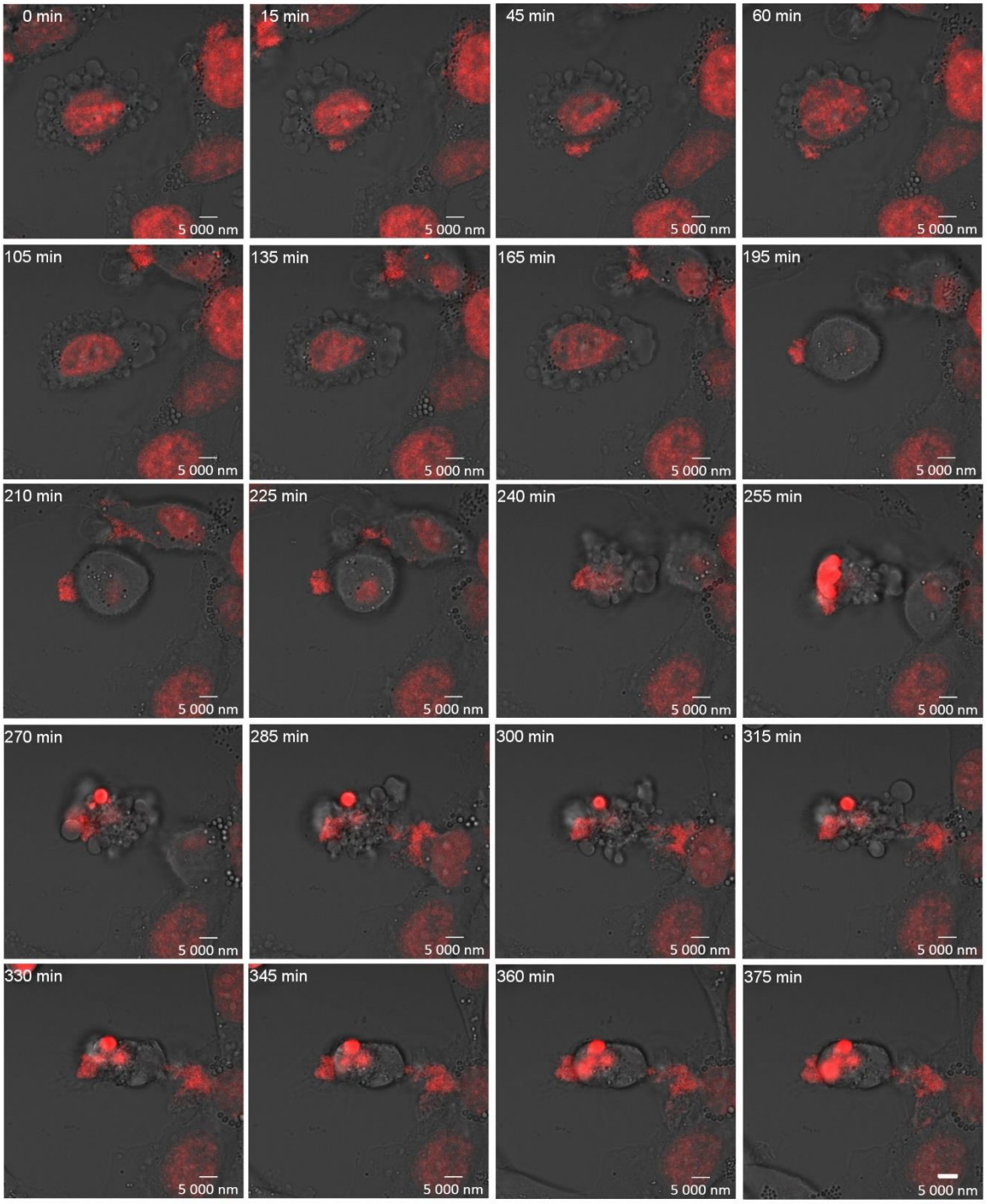
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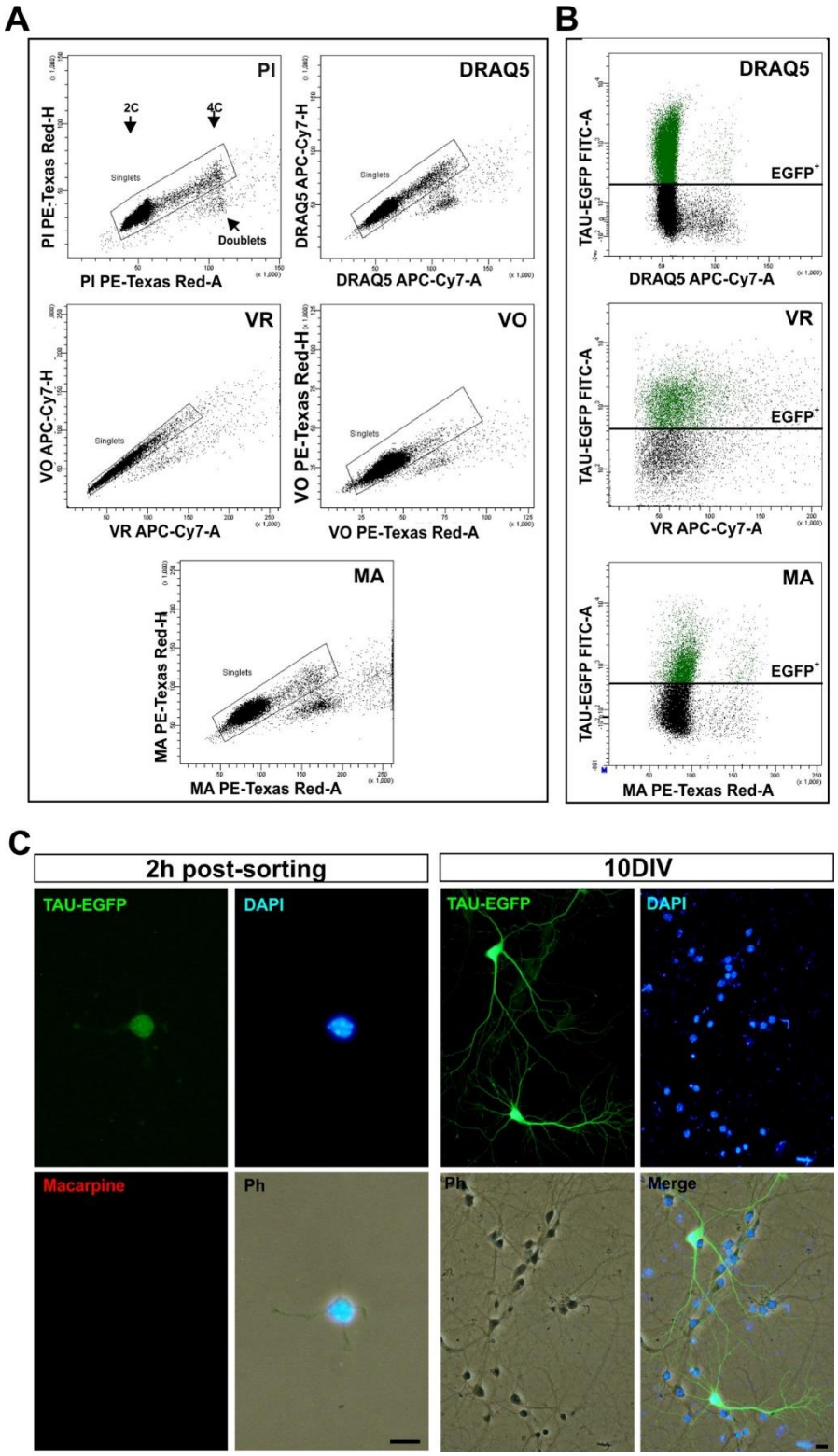
Figure 5.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Doublets
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DRAQ5
DRAQ5 APC-Cy7-H
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VR
VO APC-Cy7-H
VR APC-Cy7-A
VO
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VO PE-Texas Red-A
MA
MA PE-Texas Red-H
MA PE-Texas Red-A
B
DRAQ5
TAU-EGFP FITC-A
EGFP+
DRAQ5 APC-Cy7-A
VR
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10DIV
TAU-EGFP
DAPI
Macarpine
Ph
Merge

Figure 6.

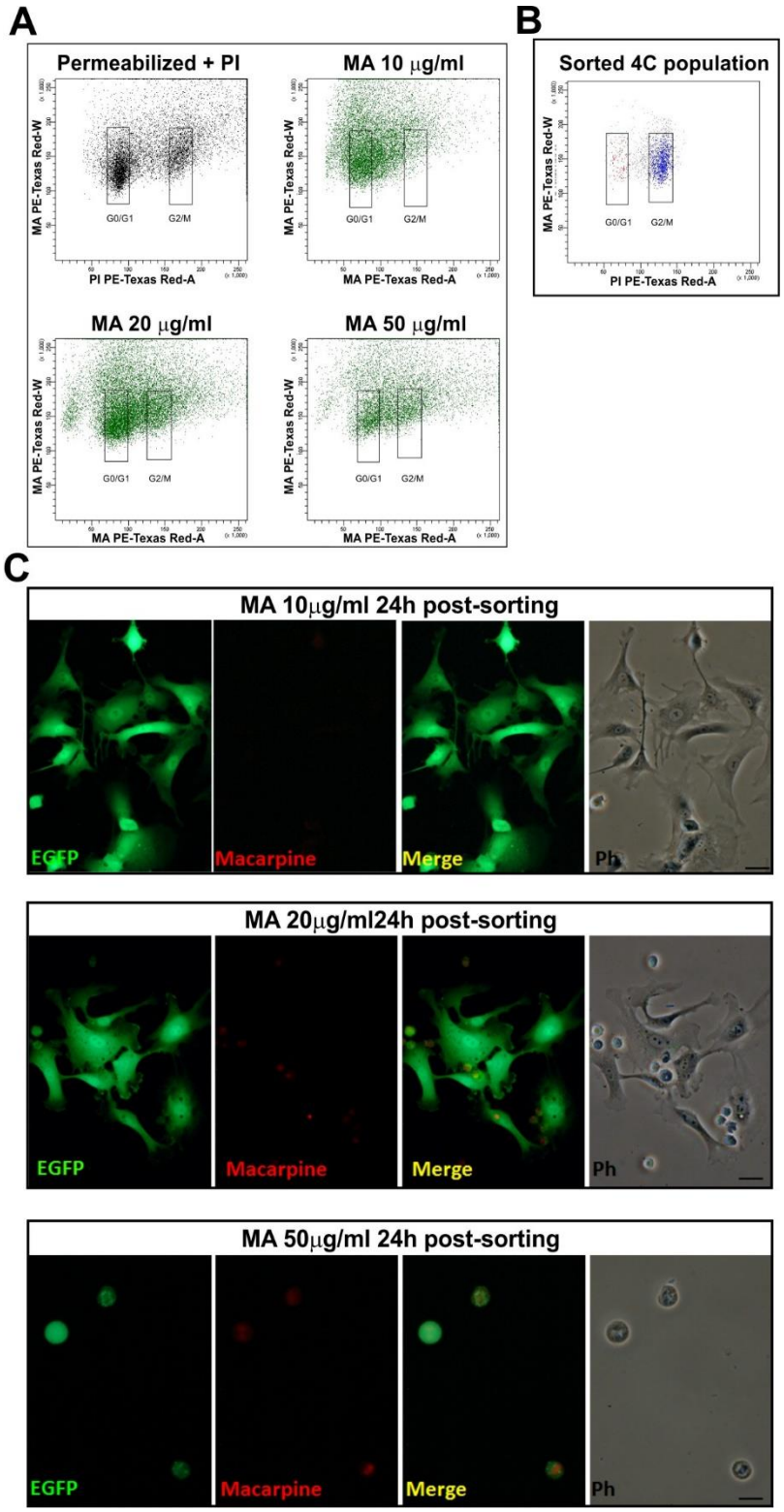
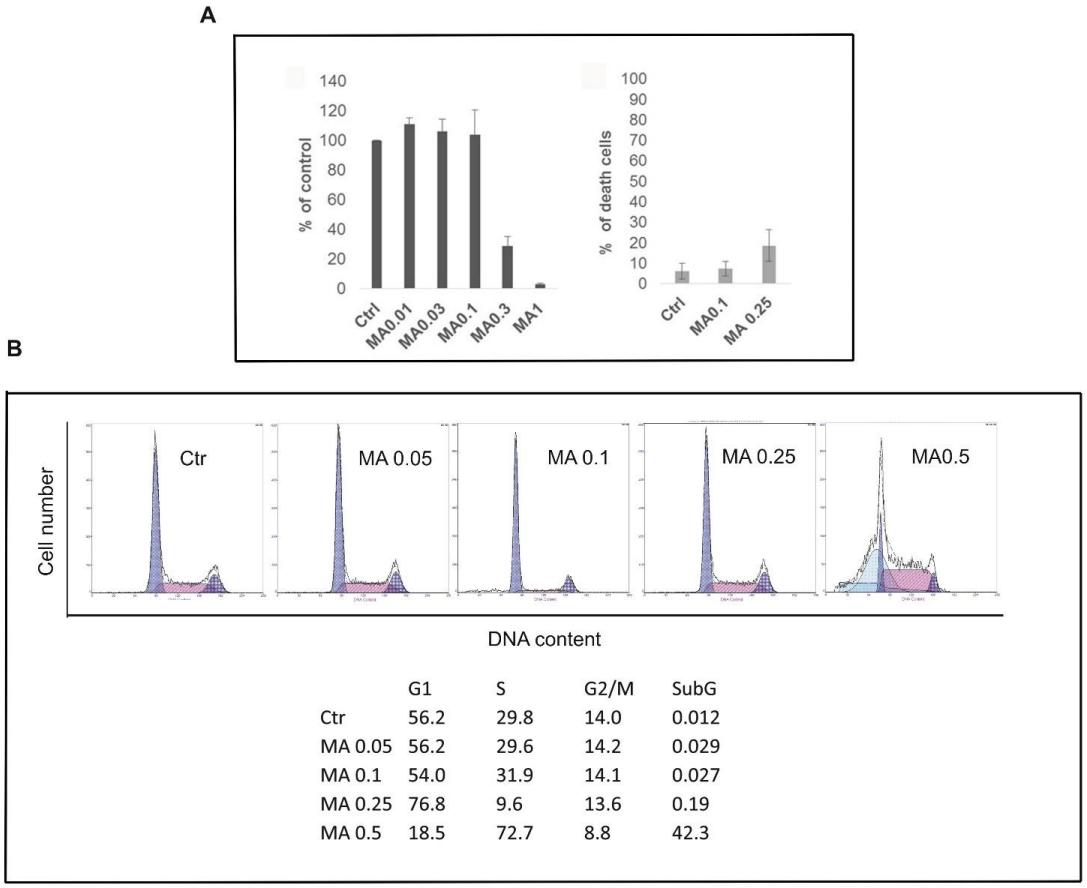

A
Permeabilized + PI
MA 10 μg/ml
MA 20 μg/ml
MA 50 μg/ml
MA PE-Texas Red-W
PI PE-Texas Red-A
MA PE-Texas Red-A
G0/G1
G2/M
B
Sorted 4C population
C
MA 10μg/ml 24h post-sorting
MA 20μg/ml24h post-sorting
MA 50μg/ml 24h post-sorting
EGFP
Macarpine
Merge
Ph

Figure 7.



	G1	S	G2/M	SubG
Ctr	56.2	29.8	14.0	0.012
MA 0.05	56.2	29.6	14.2	0.029
MA 0.1	54.0	31.9	14.1	0.027
MA 0.25	76.8	9.6	13.6	0.19
MA 0.5	18.5	72.7	8.8	42.3

Figure 8.