Exhaustive in vivo labelling of plasmid DNA with BrdU for intracellular detection in non-viral transfection of mammalian cells

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Research Article ((5784 words))

Exhaustive *in vivo* labelling of plasmid DNA with BrdU for **intracellular** detection in
non-viral transfection of mammalian cells

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Keywords:

DNA **detection**, gene delivery, 5-bromodeoxyuridine, *in vivo* labelling, transfection

Abstract

The study of the **non-viral** gene delivery process at the molecular level, e.g. during the transfection of mammalian cells, is currently limited by difficulties to specifically detect the transfected plasmid DNA within the cells. Herein we describe the *in vivo* production of 5-bromodeoxyuridine- (5-BrdU) labelled plasmid DNA by a thymine-requiring *Escherichia coli* strain leading to 92 ± 15 % BrdU incorporation while minimizing plasmid structure alteration. The labelled plasmid is produced in the milligram scale in a **two-stage** cultivation process. The relevance of this approach for plasmid DNA visualization in the field of gene delivery is demonstrated by **localising the BrdU-labelled plasmid DNA via immunodetection / fluorescence microscopy in CHO-K1 cells** after electroporation with naked, BrdU-labelled plasmid DNA and after polyfection with PEI/BrdU-labelled plasmid complexes.

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4 **Introduction**
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6 The genetic modification of mammalian cells can be achieved by a number of viral and non-
7 viral methods. Viral transfection is orders of magnitude more efficient and therefore preferred
8 in genetic medicine, i.e. for gene delivery to multicellular organisms. The typical application
9 in biotechnology, i.e. the genetic modification of eukaryotic (mammalian) cells for the crea-
10 tion of the recombinant production organism, on the other hand, relies almost exclusively on
11 non-viral methods, mainly due to a superior safety profile and the possibility to deliver much
12 larger amounts of DNA. A variety of non-viral gene delivery methods for mammalian cells
13 has been used successfully for years, including *inter alia* electroporation and transfection via
14 complexation of the DNA with the polycation PEI (polyethylenimin). However, some ques-
15 tions remain open, in particular in regard to the events between the first entry of the DNA (the
16 complexes) into the cell and the final integration of the transfected DNA into the genomic
17 DNA of the host cell. Answers to these questions could help to identify hitherto unknown
18 bottlenecks of non-viral transfection and eventually help to improve the performance of such
19 methods, thereby improving the efficiency of process development in mammalian cell culture
20 technology.

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Difficulties in the specific detection of the transfected plasmid DNA (pDNA) in the cells rep-
resent an important limitation for the study of the gene transfer process. So far, only a limited
number of studies have been reported where the transfected pDNA was directly visualized.
The majority of these studies used intercalating or fluorescent dyes for labelling the pDNA
preliminary to transfection [1, 2]. It has been recognized, however, that such labels will inevi-
tably modify the polarity of the plasmid and thus potentially alter its intracellular trafficking
as has, e.g., been shown for the Hoechst 33258 dye [3].

The thymidine analogue 5-bromodeoxyuridine (BrdU) can specifically replace the thymidine
residue in DNA molecules [4]. An immunoenzymatic detection procedure, based on the use of

a monoclonal antibody specific for 5-bromodeoxyuridine (BrdU), has been described for BrdU-labelled DNA in DNA-DNA and DNA-RNA hybridization assays involving BrdU-labelled probes [5, 6]. BrdU labelling of genomic DNA has also been used as a tool for assessing metabolic activity in mammalian cells and tissues via DNA synthesis [7] under conditions where low amounts of the label are integrated into the genomic DNA. The *in vivo* incorporation of BrdU into plasmid DNA by *E. coli* was first proposed by Kitazawa [8] leading to a substitution of approximately 80 % of T by BrdU. According to the authors, the labelled plasmid could later be used as probe for hybridization after restriction endonuclease digestion. El Ouahabi and colleagues labelled small amounts of plasmid DNA using a modified nick-translation protocol and demonstrated that BrdU-labelling can be a useful tool to visualize exogenous DNA after transfection [9].

Herein we describe an improved method for the *in vivo* production of BrdU-labelled plasmid DNA in a thymidine-requiring *Escherichia coli* strain, leading to 92 ± 15 % BrdU incorporation. Labelled plasmid DNA was produced at the milligram scale using a two-stage growth/production process. The possible relevance of this system for investigating intracellular events during gene delivery is demonstrated by visualising BrdU-labelled pDNA within the cytoplasm of Chinese Hamster Ovary (CHO) cells after electroporation and polyfection using polyethyleneimine (PEI) as transfection agent.

Materials and Methods

Materials. Chemicals were from established suppliers such as Sigma-Aldrich and used as obtained. Milli-Q™ water was used for the preparation of all aqueous solutions. PEI (branched, 25 kDa, Sigma) was prepared as 10 µM aqueous solution in Milli-Q™ water. The primary monoclonal anti-BrdU antibodies were from Invitrogen (clone IU-4) and from Sigma (clone BU33). The secondary antibodies were either a goat anti-mouse IgG conjugated to R-

PE (R-phycoerythrin, Invitrogen, immunofluorescence studies) or a rabbit anti-mouse IgG antibodies conjugated to HRP (horse radish peroxidase, Dako, dot-blot).

Fluorescence labelling of PEI

For fluorescence labelling of the transfection agent PEI, a method adapted from Godbey et al. was used [1]. For labelling with Rhodamin Red (Molecular Probes), branched PEI was dissolved at a concentration of 10 mg/mL in a 0.1 M aqueous solution of NaHCO₃. A 1-mL PEI aliquot was then transferred to a microcentrifuge tube. While shaking, 61.5 µL of the fluorochrome solution (rhodamine red X succinimidyl ester, in DMSO at 10 mg/mL) was added; thereafter shaking was continued for 1 hour in the dark at room temperature. Then, the samples were further incubated for 1 hour in the dark without agitation. The residual non-incorporated fluorochrome was removed by chloroform extraction. The labelled PEI was stored at 4 °C protected from light until use. The protocol for labelling with Cascade Blue (Molecular Probes) was similar. However, only 48.5 µL of dye (10 mg/mL in DMSO) were added, while residual dye was removed by ultrafiltration (centrifugation, 1.5 mL Microcon YM-3 tube, membrane: regenerated cellulose MCO 3000 Da, 2 washings with Milli-Q water) instead of chloroform extraction.

Plasmids

pH₂B-GFP [10], encoding for the nuclei localized green fluorescent protein (GFP), was used for the experiments. All plasmids were amplified in *E. coli chi 1776* strain, in 394 medium or in 394-BrdU medium (see below) to sufficient quantities by using standard molecular biology techniques, including harvesting and purification via Qiagen Plasmid Midi-kit (Qiagen). DNA concentration and quality were determined by OD_{260/280} ratio and by agarose gel electrophoresis (topoisomers and restriction pattern).

Bacteria strain and culture conditions. The thymidine-requiring *Escherichia coli chi-1776* (DMS 3804) strain was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). Cells were grown in '394 medium' (LB medium supplemented with 5 mM MgCl₂, 0.01 % diaminopimelic acid, 0.5 % glucose, and 40 mg/L thymidine) at 37 °C. For

BrdU labelling, 40 mg/L BrdU was added to the medium instead of the thymidine ('394-BrdU medium').

Cell culture. The Chinese Hamster Ovary cells (cell line CHO-K1, CCL-61, ATCC) were maintained in growth medium (RMPI 1640 medium supplemented with L-glutamine, penicillin/streptomycin and 10 % foetal calf serum (FCS); all from PAA laboratories) in an atmosphere of 5.0 % CO₂ and at 37 °C.

Transfection. Cells were seeded on cover slips, at 2×10^5 cells per well in 6-well plates (Greiner) 24 h before transfection. One hour before transfection, cells were rinsed with PBS (0.137 M NaCl, 1.47 mM KH₂PO₄, 2.68 mM KCl, 8.1 mM Na₂HPO₄, pH 7.4) and supplemented with fresh serum-reduced culture medium OptiMEM/Glutamax (Gibco). 3 µg plasmid DNA were diluted to 300 µL in 150 mM NaCl and vortexed. Then, 9.9 µL of PEI solution (10 µM) was added to the pDNA-NaCl solution, which was vortexed and incubated for 10 min at room temperature for complex formation. Thereafter, the PEI/DNA complexes were added to the cells (time 0) and the plates were briefly centrifuged (200 g, 5 min, 4 °C). The cells were incubated at 37 °C and 5 % CO₂ for 4 hours, at which time the medium was replaced with 2 mL of RPMI-10 % FCS. Incubation was continued until the indicated time. Note: cells that were evaluated at time points shorter than 4 hours did not receive a medium replacement after transfection.

For electroporation, the cells were suspended in cold RPMI 1640 without FCS at a density of 2×10^7 cells mL⁻¹. A volume of 0.25 mL was transferred to a sterile electroporation cuvette (Thermo Electro Corporation, 50 x 4 mm) and 20 µg plasmid DNA in 250 µL RPMI 1640 was added to the cells. After 10 min incubation at room temperature, the aliquot was electroporated using a Bio-Rad Gene Pulser-Transfection Apparatus at 500 µF and 300 V. After receiving the electric pulse, the cells were incubated in the cuvette for 10 min at 37 °C before

being transferred to 6-well plates (Greiner) and incubated until the indicated time at 37 °C in a humidified atmosphere of 5 % CO₂.

Labelling of DNA with 5-bromodeoxyuridine

A single colony of *Escherichia*

coli *chi-1776* strain containing pH₂B-GFP plasmid DNA was inoculated in 15 mL 394 medium. The culture was grown overnight under vigorous shaking (180 rpm) and the bacteria were diluted to OD₆₀₀ = 0.3 and again grown in the thymidine-containing medium at 37 °C under vigorous shaking until OD₆₀₀ = 1.3 was reached. Thereafter, the bacteria were recovered by centrifugation (10 min, 4 °C, 4300 rpm, centrifuge: Heraeus 2LR), washed once with 394-BrdU medium (resuspension/centrifugation as above), and finally resuspended in 394-BrdU medium at a OD₆₀₀ = 0.3, and grown at 37 °C under vigorous shaking overnight. After harvesting and lysing the bacteria, closed circular plasmid DNA was purified using a commercial plasmid purification kit (Qiagen) according to the manufacturer's instructions.

Dot-blot detection of BrdU For the detection of BrdU incorporated in DNA by dot-blot, a modified protocol of the method proposed by Ueda *et al.* [11] was used. Briefly, 2 µg DNA was denatured by incubation with 10 volumes of 0.4 M NaOH for 30 min at room temperature. The DNA solution was then placed on ice and neutralized by an equal volume of 1 M Tris HCl (pH 6.8). 15 to 450 ng of single-stranded neutralized DNA was spotted onto an Optitran BA-S 83 nitrocellulose membrane (Schleicher and Schuell). The filters were dried at room temperature and then baked for 2 h at 80 °C under vacuum. Afterwards, the membrane was incubated with the anti-BrdU antibody (1:2,000 dilution, clone IU-4) in TBS-T (20 mM Tris - HCl, pH 7.6, 136 mM NaCl, 0.05 % Tween 20, containing 1 % nonfat milk) for 1 h at room temperature. Excess antibody was removed by washing three times with TBS-T. The membrane was incubated with an anti-mouse IgG-HRP antibody (1: 5,000 dilution in TBS-T) for 1 h at room temperature. The membrane was then washed three times (20 min each) with TBS-T, and the substrate for alkaline phosphatase (1 mg/mL DAB (3,3-diaminobenzidine) in 50

mM Tris -HCl, pH 7.6, containing 0.6 % H₂O₂) was added for an incubation of 5 to 10 min in the dark. The enzyme reaction was stopped by washing the membrane in Milli-Q™ water.

Afterward, the blot was scanned and signal intensities analyzed using the integrated density function in ImageJ [12]. For detection of the BrdU-pDNA in the polyplex, the PEI/DNA complex was treated as described above for the naked pDNA and volumes corresponding to 15 to 450 ng pDNA were blotted on the membrane. Further treatment was as described above.

Polyplexes were in addition subjected to an 'acidic' denaturation protocol, in analogy to the one described below for intracellular DNA. For this polyplexes were incubated (after fixation in 4 % *p*-formaldehyde or not) for 30 min at room temperature in 0.5 % Triton X100 / 2 M HCl. The acid was neutralised by sufficient amounts of 1 M Tris HCl and the subsequent treatment was as described above.

In order to quantitate BrdU incorporated in the pDNA, the dot blot was calibrated as follows. Firstly a PCR product with 100 % substitution of T by BrdU was prepared as described below. In addition, the corresponding unlabelled PCR product was prepared using the GFP cDNA as template. The PCR reaction in both cases used the specific primer pair: forward primer: 5'-TCCCCCGGGGATTATTTGTAGAGCTCATCCAATGCCATGTG-3'; reverse primer: 5'-TCCCCCGGGGAGCATGGTAGCAAAGGAGAAGAACTTTTCAC-3'). 50 µL PCR mixture containing 1 x *Taq* Pol buffer, 0.20 mM of dATP, dCTP, dGTP and either dTTP or BrdUTP, and 1 U *Taq* polymerase (New England Biolabs GmbH), as well as 0.5 µM of each primer were combined with 1.0 µL DNA solution (1ng/µL). Sterile water was used as no template control. The temperature programme for the PCR consisted of one denaturation step at 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

The PCR products were purified using a commercial purification kit ("wizad plus SV mini columns" kit; Promega) according to the manufacturer's instructions. All PCR products were

evaluated by agarose gel electrophoresis after purification and the DNA concentration was estimated by measuring the absorption at 260 nm. For calibration of the dot blot, the indicated amounts (15–450 ng) of PCR product containing either thymidine or BrdU were dot-blotted on a nitrocellulose membrane (see below for the protocol) and the incorporated BrdU was immunochemically visualized. The calibration curve was prepared from 6 concentration points, each measured five times. Means and standard deviations were calculated and formed the basis for a weighed regression.

Intercellular detection of BrdU via immunofluorescence For detection of BrdU-containing DNA inside the cells, cells were washed twice with PBS and then fixed in 4 % *p*-formaldehyde/PBS for 20 min at room temperature. After fixation, cells were incubated in 0.5 % Triton X100 / 2 M HCl for 30 min at room temperature. Cells were washed three times for 5 min with PBS and incubated for 30 min at 37 °C in the blocking buffer (PBS containing 0.5 % Tween 20 and 0.5 % BSA). Then the cells were incubated with the anti-BrdU antibody (1:160 dilution; clone BU33) in blocking buffer for 1 h at RT. After three 5-min washes with PBS, the cells were incubated with the anti-mouse IgG-RPE (diluted 1:200 in blocking buffer supplemented with 1 mg/L Hoechst 33258) for 1 h at room temperature. Cells were then washed twice for 5 min with PBS-T and once with Milli-Q water. The cover slips were mounted in Prolong Gold (Invitrogen) mounting medium. Cells were observed under an epifluorescence microscope (Olympus BX51TF). In addition, images were obtained using a laser scanning confocal microscope (Leica TCS-SP) equipped with argon and neon lasers (Leica). Serial images of rhodamine fluorescence at 0.16 μm Z-intervals were recorded separately. Images were further analyzed using Bioview 3D software [13].

Results and discussion

5-BrdU incorporation into pDNA.

For pDNA labelling with 5-bromouracil (replacement of T by BrdU), the thymidine-requiring *Escherichia coli* *chi-1776* strain was to be used. Since this strain is described to preferentially grow at 30 °C [14], preliminary experiments were performed to compare the growth of this microorganism at 30 and 37 °C. In our hands, decreasing the culture temperature did not improve, but rather reduced the growth rate (data not shown). Thus, we opted for performing further experiments at 37 °C.

The *in vivo* labelling of plasmid DNA (pDNA) with the thymidine analogue BrdU has been, to our knowledge, described only once in the literature [8]. In that case, the authors used large amount of the thymidine analogue BrdU (500 mg/L culture medium) corresponding to 8- to 60-fold higher quantity than used by other groups for genomic labelling [e.g. 15, 16]. According to the authors, an incorporation of BrdU in 15 – 20% of the total DNA was achieved, corresponding to the substitution of approximately 80 % of T by BrdU.

In our hands, cultivation of T-requiring *E. coli* (strain *Escherichia coli* *chi-1776*, containing plasmid pH₂B-EGFP) for 5 to 16 hours in a medium containing BrdU (concentration range from 40 to 120 mg/L) instead of thymidine, resulted in significantly retarded growth and led to cell densities ten-fold lower than in the case of the positive control (bacteria cultivated in the presence of T), see Table 1. Bacterial growth is thus not supported in the presence of only BrdU instead of T. Moreover, even in the presence of regular amounts of thymidine (40 mg/L), the addition of more than 40 mg/L of BrdU was detrimental to bacterial growth, Table 1, most likely due to blockage of the replicative and transcriptional machinery in the presence of a surplus of BrdU over T in the medium. When 40 mg/L or less of BrdU were added in the presence of 40 mg/L T, overnight growth appeared normal, Table 1. Similarly, growth could be initiated in cultures originally grown in the presence of 40 mg/L of BrdU by the addition of 40 mg/L of thymidine, allowing the conclusion that such amounts of BrdU do not support growth, but are otherwise non-toxic for the bacteria. The discrepancy between our results and

the published data [8] may be due to the use of different strains of bacterial, as the bacterial strain used was unfortunately not specified in the previous publication. On the other hand, our results are in agreement with the data published by Hackett and Hanawalt [16], which described a marked preference of a thymidine requiring bacteria strain for thymidine over BrdU. Based on the facts that low amounts of BrdU were not toxic to the bacteria and that limited growth in the presence of BrdU was apparently possible, the following two-stage protocol was derived for *in vivo* labelling of pDNA with BrdU. The bacteria were first grown in the standard thymidine-containing medium until the mid-exponential phase was reached ('growth phase'). At this point ($OD_{600} \approx 1.3$), the bacteria were recovered and resuspended at $OD_{600} = 0.3$ in the BrdU medium ('labelling phase') where they were again allowed to grow overnight. Compared to direct cultivation in BrdU medium, this new protocol led to bacteria concentrations at the end of the culture ($OD_{600} = 2.40$), which were similar to cell densities obtained in case of cultivations in the standard thymidine-containing medium. The two-stage process was scaled up to 100 mL and allowed the preparation of up to 10 μ g of BrdU- pDNA per mL of bacterial culture at high purity ($OD_{260/280}$ ratio: 1.98). Average yields for the BrdU-labelled pDNA were thus approximately 70 % of those obtained for unlabelled pDNA prepared by the standard procedure. Finally, we confirmed by analysis on agarose gel that the incorporation of BrdU into the pDNA did not influence the content of the supercoiled topoisomer in the plasmid preparation or the restriction pattern (data not shown).

Quantitation of the BrdU-labelling index by dot-blot.

For assessment of the BrdU-labelling index of the prepared pDNA, a dot-blotting technique was adapted that had previously been described as accurate and efficient for proliferation DNA quantitation in animal tissues [11]. In particular, we amplified the GFP cDNA by PCR in the presence of BrdUTP instead of dTTP. This experimental set up assured 100 % substitution of T by BrdU in the PCR product. In addition, standard PCR products and plasmids were

produced (containing only T and no BrdU) and used as negative control. Various amounts of the BrdU-labelled PCR product (0–454 ng, 6 concentrations, each $n=5$) were dot-blotted onto nitrocellulose membranes, and the incorporated BrdU was quantified by immunoenzymatic detection, Figure 1a, to create a calibration curve, Figure 1b. The BrdU signal intensity increased in a linear fashion with an increasing amounts of dot-blotted labelled DNA (weighed linear regression: $y = 2.41 \times 10^7 \log_{10}(x) - 3.12 \times 10^8$; $R^2 = 0.970$, $n = 5$), demonstrating that this method is applicable for quantitative analyses. Standard deviations for the different concentrations – save for the very lowest concentration – were all below 15 %, which is quite satisfactory given the semi-quantitative nature of the dot blot method in general. No signal was obtained from the negative controls. In addition, this method is fairly sensitive for detecting BrdU signals, since as little as 30 ng of PCR product per dot could be reliably detected.

On the basis of these results, 0 to 454 ng of pDNA isolated from bacteria grown on BrdU medium were dot-blotted in duplicate to quantitate the substitution of T by BrdU and to calculate the BrdU-labelling index. For this purpose, the quantity of applied DNA was converted into thymidine units and the amount of BrdU-units was determined by dot-blotting and subsequent densitometric analysis. According to these measurements, the labelling protocol described in this work achieves a BrdU-labelling index of 92 ± 15 %.

Subsequently, quantitation of BrdU-pDNA compacted in polyelectrolyte complexes with the polycation PEI was attempted via the dot-blot method. However, in this case no signal was obtained. This was not due to failure of the PEI/DNA-complex to bind to the membrane, since in cases where Rhodamine-labelled PEI was used to complex the DNA, the polymer could be shown to bind quantitatively to the membrane and to stay there throughout the required manipulations. However, as Ueda *et al.* showed, the anti-BrdU antibody preferentially binds to single-stranded DNA and denaturation is therefore essential for the dot-blotting method [11].

In the protocol suggested by this author and used by us, denaturation is achieved by strongly

alkaline pH (≥ 12). Since PEI is known to have a considerable buffering capacity over a wide pH-range [17, 18], we first verified that the pH during the denaturation step was in the required pH range, which was the case. Our inability to detect pDNA could thus only have been due to a complete loss of the DNA, e.g. during the washing steps, which is difficult to imagine even though at pH 12 PEI will be mostly neutralised, i.e. less able to compact the DNA, or - quite the contrary - due to the fact that in spite of its likely denaturation, the pDNA strands remain so tightly compacted in the polyplex that they are in fact inaccessible to the antibody similar to the situation in double stranded DNA. In case of PEI-DNA polyplexes containing native DNA it has, e.g. been shown that compaction can be tight enough to prevent the insertion of intercalating dyes such as ethidium bromide. Thus, in order to exclude that our failure to detect the pDNA was due to the unsuitability of the alkaline denaturation step, we combined the dot blot assay with an acidic denaturation step similar to the one used below for the intracellular detection of BrdU-labelled pDNA, but were again unable to detect our pDNA within the polyplexes.

Intracellular detection of BrdU-labelled plasmid DNA in the cytosol of mammalian cells

In order to investigate the potential of the BrdU-labelling method for localising the DNA inside mammalian cells, naked BrdU-pDNA, namely BrdU-labelled pH₂B-EGFP, was electroporated into CHO cells. Three hours *post* electroporation, the cells were fixed with 4 % *p*-formaldehyde, and treated with a permeabilisation solution (0.5 % Triton X100 / 2 M HCl), prior to staining with the anti-BrdU antibody/detection antibody pair (anti-BrdU antibody; clone BU33, detection antibody: anti-mouse IgG-RPE). Then the preparation was analysed by fluorescent microscopy. Positive staining appeared in the cytoplasm, see e.g. Figure 2, panel B. To verify the specificity of the detection for BrdU-labelled pDNA, several controls of the protocol were carried out. When the acid treatment or the primary antibody was omitted, no fluorescence could be detected in the cell. Likewise, non-transfected cells or cells transfected

with non-labelled plasmid DNA did not give rise to any specific fluorescence signal. To further confirm the specificity of the anti-BrdU antibody detection, BrdU was incorporated into the nuclear DNA of the CHO cells by metabolic labelling and the cells were stained as described above. In this case, the fluorescence was found exclusively within the nuclei.

Concerning the localisation of the electroporated BrdU-pDNA, Figure 2, fluorescence was associated with aggregates of homogeneous size that might represent some accumulation of the labelled pDNA within small vesicles. Additionally, larger aggregates could be seen, indicated by an arrow in Figure 2, panel B, which could typically be resolved into several small aggregates by analysing the slide with phase contrast. In none of the experiments with cells 3 h *post* electroporation was BrdU-labelled pDNA ever found inside the nucleus. Analyses of cells 6 h, 12 h, and 24 h *post* electroporation also gave no evidence for the presence of BrdU-labelled pDNA in the nuclei. While this could indicate an inability of the labelled pDNA to enter the nucleus, it is in view of the generally low efficiency of non-viral transfection methods more likely that the concentration of labelled pDNA inside the nucleus was simply below the detection limit of our method. It is also possible that the pDNA in the nuclei becomes masked to the antibody, e.g. due to an interaction with chromatin or some components of the transcription machinery. Incidentally, the one other work published on using BrdU-labelled plasmid DNA also failed to detect the pDNA inside the nucleus [9]. In order to gain additional information on the cellular localisation of the BrdU-pDNA, we re-analysed the slides with a laser confocal microscope (LSM). The LSM-analysis confirmed the exclusive cytoplasmic localisation of the plasmid, Figure 3, panel A.

The indirect proof of entry into the nucleus via the expression of the reporter gene was also not possible in case of the BrdU-labelled plasmid, since expression was not observed in these cases. However, when the unlabelled plasmid was used under otherwise similar transfection conditions, expression of the reporter gene (GFP) was seen as early as two hours post electro-

poration. The pronounced similarity between thymidine and the analogue BrdU argues against major differences in nuclear uptake of the two types of polynucleotide. In our opinion the lack of expression in case of the BrdU-labelled pDNA is more likely due to an incompatibility with the transcription machinery, as discussed above for the lack of bacterial growth in a culture medium supplemented with BrdU instead of thymidine.

In order to localise the pDNA in the cells after polyfection, BrdU-pDNA was complexed with PEI and fluorescent dye-labelled PEI respectively and incubated with the CHO cells for various times (ranging from 0.3 to 3 h). Since *in vitro* we had been unable to detect BrdU-labelled pDNA in the polyplexes, we expected to be reduced to seeing only the labelled PEI inside the cells, not the pDNA. To our surprise however, intracellular detection of BrdU-pDNA after polyfection was possible, Figure 2, panel D, albeit at a weaker signal than in case of the naked BrdU-pDNA after electroporation, Figure 2, panel B. This may be due to the fact that a different primary and secondary antibody pair was used in the dot blot and the intracellular analysis of the polyplexes. More likely, however, is a general destabilisation of the complex in the cytosol under transfection conditions leading to a better exposure of the BrdU in the pDNA to the antibody. The fact that it was not the *p*-formaldehyde fixation / cell permeabilisation step that lead to the difference in detectability was ascertained by repeating the dot blot analysis of the polyplexes including a mock fixation step (in combination with acidic denaturation / 'permeabilisation'). Again no signal was obtained.

Finally, in order to simultaneously visualize both the transfection agent PEI and the pDNA, CHO cells were incubated with polyplexes made from cascade-blue-labelled PEI and BrdU-pDNA. When the fluorescence distribution inside the cells was analyzed by fluorescence microscopy, PEI was indicated by fluorescent light in the blue region, while pDNA gave rise to red fluorescence. However, in these experiments no clear evidence for co-localization of the two molecules could yet be found, in particular not in the aggregates around the nuclei. It re-

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mains to be seen whether this is indeed indicative of complex dissociation in the cytosol or an artefact produced by the experimental conditions, in particular during fixation and denaturation.

Conclusions

Exhaustive labelling of *in vivo* synthesized pDNA with the thymidine analogue 5-bromodeoxyuridine- (5-BrdU) is possible using the two-stage protocol outlined in this contribution. The procedure leads to high T-substitution and has minimal influence on the structure and the restriction map of the plasmid product. Purification procedures are the same as those for non-labelled pDNA. The method contains no step that would prevent scale up to the bioreactor scale and hence constitutes a cheap and easy way of producing large quantities of labelled pDNA. This is a considerable improvement, e.g., in comparison to the nick-translation method proposed by El Ouahabi *et al.* [9] for the same purpose.

Since BrdU is a close T-analogue, BrdU-labelled pDNA can be used to investigate intracellular localisation of DNA during non-viral transfection of mammalian cells with a minimum of bias. Moreover, the detection based on a R-PE-conjugated anti-BrdU monoclonal antibody is simple and highly specific for the target DNA. Any non-labelled DNA present in the sample would therefore not be detected and its presence could not interfere with the interpretation of the results. In addition, BrdU-labelling overcomes the common problems linked with direct labelling, e.g., with fluorescent dyes; such as the alteration of the structural (supercoiled) and physico-chemical (hydrophobicity) properties of the plasmid DNA. The sensitivity of the method is comparable to those based on of fluorescent nucleic acid labelling systems. In spite of its similar structure, however, BrdU-labelled pDNA is apparently not compatible with the transcription machinery of the cells, as the corresponding gene product normally was not detected in cultures transfected with the labelled pDNA.

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For Peer Review

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Figure legends

Figure 1: Calibration of the dot-blot quantitation for 5-bromo-2-deoxyuridine (BrdU)-incorporation *in vivo*. A: various amounts (15–450 ng) of pDNA or PCR product containing either thymidine or BrdU were dot-blotted on a nitrocellulose membrane, and the incorporated BrdU was immunochemically visualized. *Lane 1*, BrdU-labelled pDNA; *lane 2*, unlabelled pDNA; *lane 3*, BrdU-labelled PCR product; *lane 4*, unlabelled PCR product; *lane 5*, BrdU-labelled pDNA complexed with PEI; *lane 6*, BrdU-labelled pDNA complexed with PEI-rhodamine; *lane 7*, unlabelled pDNA complexed with PEI; *lane 8*, unlabelled pDNA complexed with PEI-rhodamine.

B: Calibration curve created from densitometric analysis of the calibration dot blots (weighted linear regression: $y = 2.41 \times 10^7 \log_{10}(x) - 3.12 \times 10^8$; $R^2 = 0.970$; $n = 5$)

Figure 2: Intracellular distribution of BrdU-labelled plasmids after transfection. Panels A, B: CHO 3 h **post** electroporation, panels C, D: CHO cells 3 h after transfection with PEI. For the measurement cells were fixated with *p*-formaldehyde and incubated with anti-BrdU antibody (panels B, D). The nuclei were counterstained with Hoechst 33258 (panels A, C). The arrow indicates large aggregates, which could typically be resolved into several small aggregates by analysing the slide with phase contrast.

Figure 3: Representative single confocal slices of transfected CHO cells. The plasmids were visualized by immunofluorescence (anti-BrdU antibody (clone BU33)). Panel A: CHO cells after electroporation; panel B: CHO after PEI-mediated transfection. Each bar represents 20 μm .

Tables

Table 1

Optical densities, maximum growth rates, and doubling times determined after 5 hours of growth in the indicated growth medium (LB medium supplemented with 5 mM MgCl₂, 0.01 % Diaminopimelic acid, 0.5 % glucose, and 40 mg/L thymidine or BrdU respectively). Starting OD₆₀₀ in all cases was 0.02. Pre-cultures were all in the presence of thymidine.

Composition of the growth medium		End OD ₆₀₀	μ _{max} (h ⁻¹)	t _d (h)
Thymidine	40 mg/L	2.40	1.20	0.58
Bromodeoxyuridine	40 mg/L	0.21	0.51	1.37
	80 mg/L	0.19	0.51	1.37
	120 mg/L	0.21	0.44	1.57
Thymidine + Bromodeoxyuridine*	0 mg/L	2.44	n.d.	n.d.
	40 mg/L	2.33	n.d.	n.d.
	80 mg/L	0.1	n.d.	n.d.
	120 mg/L	0.2	n.d.	n.d.

*: medium contained 40 mg/L thymidine and incubation was overnight.

n.d. not determined

A

Figure 1A (Jérôme et al.)

Figure 1B (Jérôme et al.)

Figure 2 (Jérôme et al.)

Figure 3 (Jérôme et al.)