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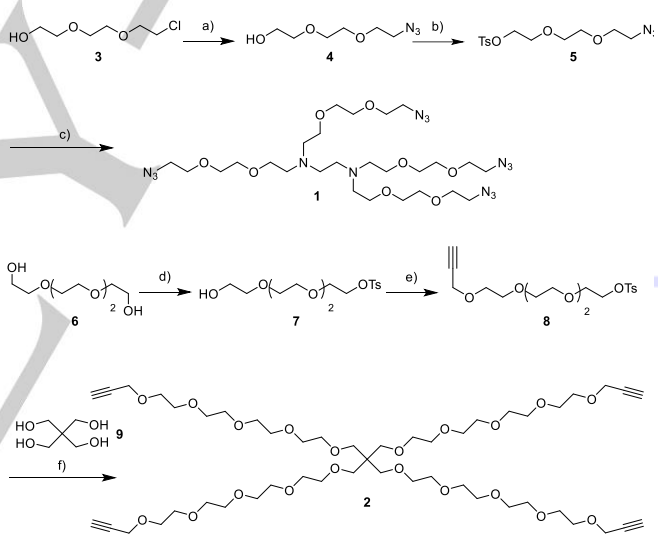
Dendrimer-based signal amplification of click-labelled DNA *in situ*

Nada Raddaoui[#], Samuele Stazzoni[#], Leonhard Möckl, Bastien Viverge, Florian Geiger, Hanna Engelke, Christoph Bräuchle and Thomas Carell*[a]

The in vivo incorporation of alkyne modified bases into the genome of cells is today the basis for efficient detection of cell proliferation. Cells are grown in the presence of ethinyl-dU (EdU), fixed and permeabilized. The incorporated alkynes are then efficiently detected using azide-containing fluorophores and the Cu(I) catalyzed alkyne-azide click reaction. In a world where constant improvement of the sensitivity of a given method is driving diagnostic advancement, we developed azide and alkyne modified dendrimers that allow to establish sandwich-type detection assays that show significantly improved signal intensities and signal to noise ratios far beyond of what is currently possible.

The proliferation rate of cells is a key parameter that requires in many contexts precise determination.[1] Cell proliferation assays help for example routinely to evaluate the toxicity of compounds in the framework of the development of new pharmaceuticals.[2] Also in cancer diagnostics, it is required to measure the proliferation of cells with high precision.[3] Particularly in this field highest sensitivity is desired in order to detect at best even single cancer cells in a patient sample. Today the most precise way to measure cell proliferation is to culture the cells in the presence of C5-ethinyl-dU (EdU), which is incorporated into the genome of proliferating cells as a typical anti-metabolite.[4] The amount of incorporated EdU is subsequently measured by reacting the alkynes within the DNA with azido-modified fluorescent dyes using the Cu(I) catalysed alkyne-azide click reaction[5] and detected using fluorescent microscopy.[6] This reactions proceed on DNA with extreme efficiency likely because the Cu(I) is loosely pre-coordinated to the electron rich centers at the nucleobases.[6] This technology is used in established commercially available kits (EdU-Click kit from baseclick, Click-iT from Thermo Fisher). However in all available methods, the sensitivity is limited by the number of alkynes, which are incorporated during the culturing phase of the experiment in the presence of EdU (one alkyne). This creates the problem that slowly proliferating, but still cancerogenous cells often escape detection.[7] We report here a sandwich-type approach with alkyne and azide containing dendrimers **1** and **2** (Scheme 1) that allows significant chemical signal amplification. The method was shown to provide unprecedented detection sensitivities of proliferating cells. The synthesis of the needed amplifying tetraazide/alkyne molecules **1** and **2** is depicted in Scheme 1 (and S1, S2). In both cases, the principle design idea was to stay as close as possible to polyethyleneglycol based structures because of the needed high solubility in water. Starting point towards **1** is the ethylene glycol derivative **3**, which we converted first into the azide **4**. The hydroxyl group was subsequently tosylated to **5** to enable the fourfold substitution reaction with ethylenediamine to give the desired tetraazide compound **1**.



Scheme 1. Synthesis of the ethylene glycol based tetraazide **1** and of the tetraalkyne **2** needed for the study. Reagents and conditions: a) NaN_3, DMF, 90°C, o/n, 96%. b) TsCl, NEt_3, CH_2Cl_2, o/n, 90%. c) ethylenediamine, **5**, KOH, LiBr, DMF, 60°C, o/n, 63%. d) TsCl, NEt_3, THF, rt, o/n, 92%. e) propargyl bromide, NaH, THF, 0°C to rt, 82%. f) **8**, K_2CO_3, acetone, 80°C, o/n, 31%.

The synthesis of the tetraalkyne **2** started with the ethyleneglycol derivative **6**, which was monotosylated in excellent yield to provide **7**. Reaction with propargyl bromide furnished compound **8**, which was used for a fourfold substitution reaction with pentaerythritol **9** to give the dendrimer **2**. Both compound **1** and **2** were subsequently purified by flash column chromatography.

In a first approach to amplify the cell proliferation signal, we used the polyethyleneglycol based tetraazide molecule **1** as shown in Figure 1A and S3. For the experiments, we grew HeLa cells in µ-slides in the presence of 10 µM EdU for 2 h. The medium was removed and 3.7% formaldehyde in phosphate buffered saline supplemented with 0,02% Tween (1x PBS-T) buffer was added to fix the cells. After two time washing with 1x PBS-T, the cells were permeabilized with 0.5% Triton X-100 in 1x PBS-T for 20 min at

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room temperature (RT). We then added the click-cocktails successively. In one experiment, we just added the Tamra-azide **10** (20 μM) as a positive control. In the other experiment, we performed a first reaction with the dendrimer **1** (20 μM) for 1 h followed by a second click reaction with the Tamra-alkyne **11** (5 μM) for 30 minutes (For more detailed characterization of the reaction products, determined with a symplified model using synthetic oligonucleotides see SI). In both experiments, we washed the fixed cells twice with 3% BSA in PBS buffer. In the dendrimer amplified experiment with the Tamra-alkyne **11** we noted an unusually high background even after these intensive washing steps. Screening for appropriate washing conditions showed that best results were obtained when we washed with a solution of guanidinium isocyanate (Fig. S4). We then determined the Tamra fluorescence using a fluorescence microscope. The data are depicted in Fig. 1B. Clearly visible is that the prior click with the dendrimer-azide **1** furnished a six-fold increase of the fluorescence signal. This was particularly visible in the fluorescence microscopic evaluation of the click-modified cells (Fig. 1C).

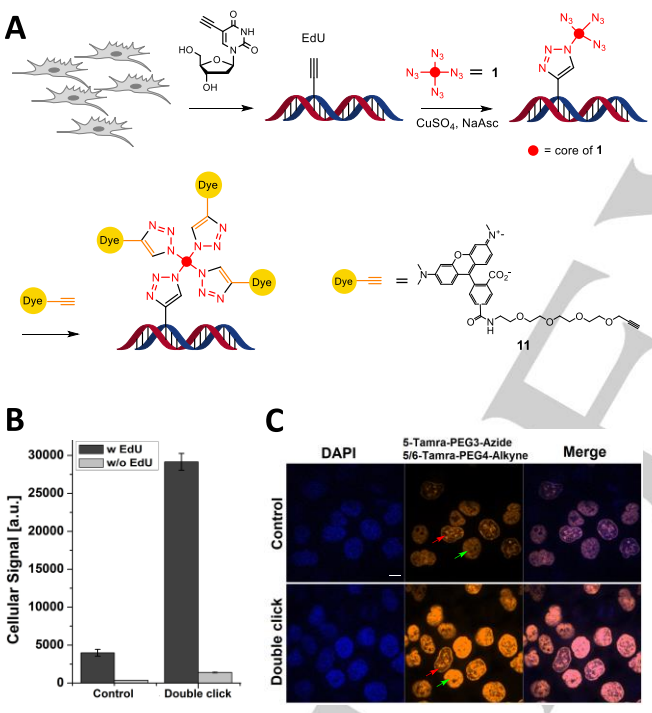

Figure 1. A) Depiction of the single dendrimer (double click) amplified cell proliferation assay. Cells were grown in the presence of 5-ethinyl-dU. The cells were fixed and the present alkynes reacted with a tetraazide-dendrimer **1** in the presence of Cu(I) (double click). The multiple azide containing DNA is then detected *in situ* with an alkyne-modified dye **11** using again the Cu(I) catalyzed click reaction. **B)** The control experiment is performed using the dendrimer-free standard proliferation assay with **10**. Double click shows data after dendrimer amplification. **C)** Fluorescence microscopy pictures of cells detected with the standard assay as control (top) and after dendrimer amplification (bottom). Red arrays show cells in the early S-phase with partial EdU incorporation. Green arrows show cells in late S-phase, where the DNA synthesis is almost finished and EdU is incorporated into the whole genome. Scale bars, 20 μm.

We noted that after extensive washing with guanidinium isocyanate, the background was reduced but steadily higher than in the non-dendrimer experiments. We speculated that this background problem may be caused by the dye-alkyne **11** possibly because alkynes are known to react to some extent with nucleophiles. Thiol-containing nucleophiles are abundantly present in cells. Control experiments performed without the addition of the tetraazide **1** confirmed this hypothesis and showed a still high background signal even without using our dendrimers (Fig. S4).

In order to solve the background problem and to further increase the sensitivity (signal to noise ratio) of detection, we experimented next with a double-dendrimer approach (Fig. 2) where we first reacted the fixed DNA with the tetraazide **1**, followed by an additional click reaction with the tetraalkyne-dendrimer **2**. This was then followed by a final click reaction with the Tamra-azide **10** (Fig. S5). In this way, we planned to circumvent the use of the problematic dye-alkynes.

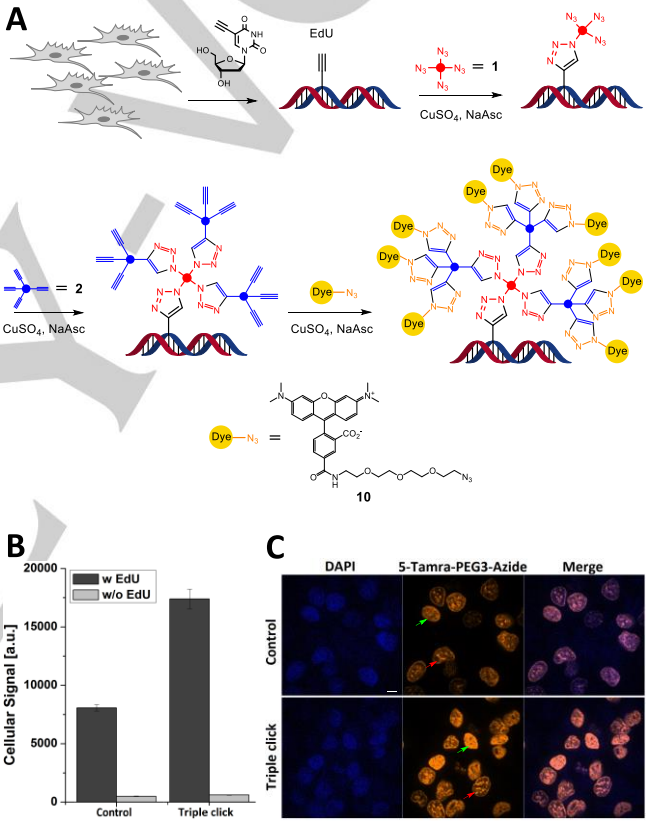

Figure 2. A) Depiction of the double dendrimer (triple click) amplified cell proliferation assay. Cells were grown in the presence of 5-ethinyl-dU. After fixation and permeabilization, the present alkynes are first reacted with the tetraazide-dendrimer **1** in the presence Cu(I). The multiple azide containing DNA is then reacted with the tetraalkyne **2**. The so double modified DNA (triple click) is finally detected with an azide-modified dye **10** using again the Cu(I) catalyzed click reaction. **B)** The control experiment is performed using the dendrimer-free standard proliferation assay. Triple click shows data after double dendrimer amplification. **C)** Fluorescence microscopy pictures of cells detected with the standard EdU assay as control (top) and after double dendrimer amplification with triple click (bottom). Red arrows show cells in the early S-phase. Green arrows show cells in late S-phase. Scale bars, 20 μm.

For this experiment, we again cultured HeLa cells in μ-slides in the presence of 10 μM EdU for 2 h. The medium was removed and 3.7% formaldehyde in 1x PBS-T was again added to fix the cells. We washed the cells two times with 1x PBS-T and permeabilized the cells with 0.5% Triton in 1x PBS-T for 20 min at RT. We then added the click-cocktails successively: First we

added Cu(I) and tetraazide **1**. We washed the cells twice with a 0.2 M acetate buffer pH 4.7 followed by two washing steps with 1x PBS-T and performed subsequently the second click reaction with tetraalkyne **2** and Cu(I) for 1 h. The cells were again washed twice with 1x PBS-T. Finally we added Cu(I) and the Tamra-azide **10** to the cells and allowed the final click cocktail to penetrate the cells for 30 minutes. After again two time washing with guanidinium isocyanate we studied the cells by fluorescence microscopy. This time the experiment was a full success. We detected a strongly reduced background, not higher than in the control experiment with just EdU (Fig. 2B,C). The obtained fluorescence signal was highly improved by a factor of 2.5. Most importantly, the direct inspection of the cells by fluorescence microscopy shows a strongly improved signal to noise ratio (Fig. 2C, S5).

Next, the new single and double dendrimer based methods were applied for high throughput screening (HTS). This method is the most widely used tool not only for the development of new pharmaceuticals compounds but also needed for the measurement of the response of cells to different nutrients, mitogens, cytokines, growth factors and toxic agents[8]. With the signal amplification provided by our dendrimers, we were able to detect a strong, specific signal even when only a very small number of cells like just 100 cells were present per well (Fig. 3). This is a significant improvement over contemporary methods that need 500 to 1000 cells per well, which allows now the reliable detection of small number of proliferating cells that otherwise escape staining and detection. What we noted, however, is a reduction of the signal intensity in the double-dendrimer approach, which is likely due to self-quenching of the then densly packed fluorophores. To solve this, optimization of the dendrimers is now required.

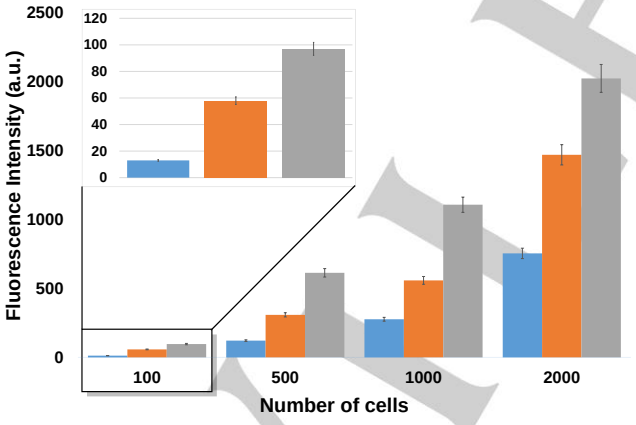

Figure 3: Application of the single and double dendrimer amplified assay in high throughput screening. Cells were grown on microplate with different densities (100, 500, 1000 and 2000 cells/well) and incubated for 2 h with 5-ethinyl-dU at 37°C. Negative control cells were grown without EdU labeling. The cells were fixed and permeablized and the present alkynes reacted with a tetraazide dendrimer **1** and Tamra-alkyne (double click, gray bars) or with tetraazide **1**, tetraalkyne **2** and Tamra-azide (triple click, orange bars) in the presence of Cu(I) *in situ*. Positive control cells were reacted with Tamra-azide in presence of Cu(I) (control, blue bars). The cellular signal of duplicate samples was measured with a Tecan microplate reader. After subtraction of the background fluorescence, it was possible to detect a stronger signal even with only 100 cells. Blue: Standard click protocol. Grey: Single dendrimer approach with dendrimer **1**. Orange: Double dendrimer approach with the dendrimers **1** and **2**.

In summary, click-based detection of cell proliferation is today state-of-art technology. We show here that by using dendrimer-type tetraazide (**1**) and dendrimer-type tetraalkyne (**2**) compounds sandwich type detection assays can be established that yield strongly improved signal intensities with low background giving higher signal to noise ratios for imaging and high throughput content assays. We expect that the so improved cell proliferation assay will be able to detect either slowly or even single proliferating cancer cells with unprecedented sensitivity.

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Keywords: click chemistry • cell proliferation • dendrimer • fluorescence microscopy • high throughput screening

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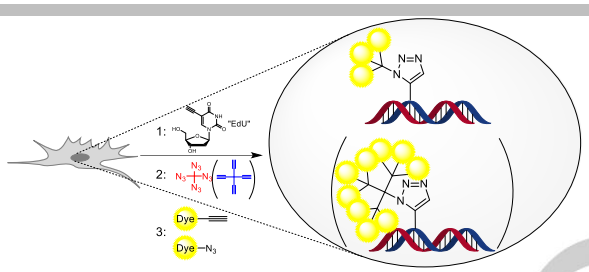
Finding a needle in a haystack: A new method to detect proliferating cells *in situ* using multiple consecutive click reactions with dendrimeric molecules and clickable dyes is presented, that allows to reach outstanding sensitivities



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