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Express FRET Labeling and Analysis of Phagocytic Clearance

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Abstract

Lysosomal DNase II in phagocytic digestion produces DNA ends with 3'PO₄/5'OH, which differ from those created in apoptotic DNA fragmentation, and can be used to label phagocytic clearance of cell death. Here, we describe the use of these specific DNA ends as selective markers of phagocytic reaction in cell suspensions. The approach does not require cell fixation. It selectively labels blunt-ended DNA breaks with terminal 5'OH. The detection is performed by ultra-fast FRET probes in a single step, closed-tube procedure. It takes 3 min and is signaled by fluorescence. The full step-by-step protocol is presented as well as instructions on analysis and representation of the results.

The described DNA-end-based phagocytosis marker and the new rapid FRET assay can be useful in studies of phagocytosis, apoptosis and in immune system assessments.

Keywords

FRET probes; Nanoblinkers; Phagocytic digestion of DNA; 5'OH DNA breaks labeling; Phagocytosis; Phagolysosomes; Apoptotic cell clearance; Express detection of DNase II cleavage; 5'OH DNA probes

1 Introduction

Apoptotic fragmentation and phagocytic digestion of DNA are the only two processes in cells which can specifically generate large numbers of blunt-ended DNA breaks. However, these two pools of breaks differ principally in the distribution of their functional end-groups. Apoptotic nucleases produce DNA breaks with 3'OH/5'PO₄, while lysosomal DNase II in phagocytic digestion makes the inverted 3'PO₄/5'OH configuration [1, 2].

In another report in this volume, we described an approach for fast and specific labeling of such DNase II-type breaks in situ, in fixed tissue sections [3]. The procedure permitted selective visualization of active phagocytes performing clearance of apoptotic cells. On a subcellular level, the in situ assay labeled phagolysosomes in the cytoplasm of the cells that engulfed apoptotic nuclei. These cellular organelles are responsible for the actual execution of phagocytic degradation of apoptotic cell corpses, and are the final sites where engulfed DNA material is destroyed by DNase II [4, 5].

Here, we expand the labeling of DNase II-type cleavage to live cell suspensions, such as cultured non-adherent cells. The technique can also be adapted to noncultured cells, such as those obtained in blood tests, for example peripheral blood mononuclear cells. Moreover, the procedure can be used with adherent cells after they are put in suspension.

The approach detects blunt-ended DNA breaks with terminal 5'OH, a general marker of phagocytic DNA digestion [4, 6]. Therefore, it can label all the various cell types participating in phagocytic clearance.

By being a FRET-based technology, this assay differs significantly from the previously described in situ labeling techniques [3, 4]. This is because FRET probes, which can work in a homogenous cell culture assay, have different requirements as compared to the in situ probes for tissue sections.

In a homogenous closed-tube format, the unreacted probe cannot be washed out from the reaction mix making it difficult to determine the amount of probe which actually reacted with its target. To overcome this limitation we developed a new type of FRET probes termed nanoblinkers, which do not require washing steps as they indicate detection of their targets by changing their fluorescence color. Specifically, the FRET probes described in this presentation signal the detection of specific DNA breaks by changing their fluorescence emission maxima from 580 to 525 nm (i.e., from red to green).

The general structure of the nanoblinker FRET probe and its mode of action are presented in Fig. 1. The figure shows that the probe is a dual-hairpin oligonucleotide with two fluorophores (FRET donor and acceptor) positioned in close proximity to enable FRET (*see Note 1*).

The actual oligonucleotide probe that we use in the described protocol is a 38-mer dual-hairpin labeled by FAM-TAMRA. It contains CCCTT3' sequence recognized by the TOPO enzyme. The enzyme specifically attaches to this site and makes a single cut at its 3' end (CCCTT ↓3'). This breaks the dual-hairpin into two separate hairpins, one carrying the enzyme and another enzyme-free (Fig. 1). The repetitive cleavage-religation permits separation and random re-association of the hairpins. This oscillating system is a specific detector of 5'OH DNA breaks because the length of the donor fluorescence phase, when the hairpins are separated, radically increases in their presence. A blunt-ended 5' OH DNA break represents the selective alternative target for the acceptor-carrying hairpin with bound TOPO, which can ligate to it instead of the donor-labeled hairpin [4, 8]. This interrupts FRET, shifts fluorescence emission from acceptor to donor (i.e., probe fluorescence changes from red to green), thus signaling detection of DNA damage (Fig. 2).

The nanoblinker FRET probe can uncover DNA damage in the homogenous assay format without the need to remove the unreacted probe. The assay is sensitive and in tests could distinguish between apoptotic and necrotic cell engulfment, based on the intensity of phagocytic clearance [7] (*see Note 3*).

Here, we present the complete protocol of this ultra-fast FRET technique applicable for detection of DNase II-type breaks in suspensions of unfixed, live cells. With its labeling step requiring only 3 min and no need for cell fixation, the assay provides the most rapid evaluation of specific DNA breaks. The nanoblinker FRET probe reacts exclusively with 5'OH blunt-ended DNA breaks. The detection is not affected by the presence of other types of DNA damage, beyond the target breaks of DNase II-type [7].

The described protocol was extensively used in studies of phagocytic J774A.1 cells clearing apoptotic and necrotic U87 cells [7]. The assay does not change when any other cell types are used, but in some cases it might need an adjustment.

2 Materials

1. Phagocytic J774A.1 cells from ATCC (TIB-67).
2. U87 cells from ATCC (HTB-14).
3. FRET Oligo—a 38-mer oligonucleotide dual-labeled with FAM and TAMRA:5′-AAGGGT(**TAMRA**)CCTGCTGCAGGACCCTTAACGCATTATGCGT(**FAM**)T-3′.
4. Test Oligo—a 21-mer hairpin used to emulate the blunt-ended DNase II breaks in positive control solution tests: 5′-GCGCTAGACCTGGTCTAGCGC-3′.

All oligonucleotides synthesized and PAGE purified by IDT, Coralville, IA. On receipt dilute oligonucleotides with bidistilled water to 100 pmol/μL concentration and store at −20 °C, protected from light.
5. Vaccinia DNA topoisomerase I—3000 U/μL (Millipore) (*see Note 4*).
6. 100 mM Tris-HCl, pH 7.4.
7. Scanning spectrofluorometer (Tecan Safire 2 or similar).
8. APO HTS 3/7 Caspase Detection kit (Cell Technology, Inc.). This kit is used for several purposes in the assay. First, it helps to confirm the apoptotic morphology of the cells. Second, it provides the cell lysis buffer that is also used for the disruption of cellular membranes to make the cellular contents available for the analysis by the nanoblinkers (*see Note 5*).

3 Method

3.1 Induction of Apoptosis, Necrosis, and Phagocytosis

1. Induce apoptosis in cultured U87 cells: incubate at 42 °C for 30 min, then return cells to 37 °C for 18 h.

Induce necrosis in cultured U87 cells by incubating cells at 65 °C for 10 min. Then return cells to 37 °C for 18 h.
2. Verify apoptosis and necrosis by using the APO HTS 3/7 Caspase Detection kit and morphologically by fluorescence microscopy using DAPI staining (*see Note 6*).
3. Induce phagocytosis by combining treated U87 cultures (200 cells/well) with cultured J774A.1 macrophages (20 cells/well) for 18 h at 37 °C, 5% CO₂ (*see Note 7*).

3.2 Phagocytosis Assessment with Nanoblinker FRET Probes

1. After the 18 h-long incubation place the combined macrophage and U87 cell cultures (20 macrophages/200 U87 cells) and all negative controls in the cell lysis buffer and vortex for 30 s to rupture cellular membranes (*see Note 5*).
2. Aliquot all samples into a 96-well plate.
3. Assemble nanoblinkers in 100 mM Tris-HCl, pH 7.4 by combining 50 pmol FRET Oligo and 50 pmol vaccinia topoisomerase I. Incubate for 1 min at room temperature. Add 20 μ L of prepared nanoblinkers (50 pmol total) to 80 μ L (20 macrophages/200 U87 cells) of ruptured cells diluted in 100 mM Tris-HCl, pH 7.4 in a 96-well plate. The final concentration of nanoblinkers in the wells will be 500 fmol/ μ L in 100 μ L total volume (*see Notes 2 and 7*).
4. Incubate for 3 min at room temperature, protected from light. For the incubation the plate can be placed in the spectrofluorometer ready for the measurements (*see Note 8*).
5. Immediately after the incubation simultaneously measure both donor (525 nm) and acceptor (580 nm) emissions using 488 nm excitation directly in the 96-well plate. Use the obtained values for the assessment of DNase II-type breaks in the samples (*see Note 9*). Compare experimental results with controls present in the same 96-well plate (i.e., nanoblinker probe signal without 5'OH DNA breaks) (*see Note 7*).

4 Notes

1. In FRET the rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor fluorophores. Therefore, the efficiency of the transfer very rapidly declines with distance. For each specific donor-acceptor pair a distance (in $\text{\AA}$) at which energy transfer is 50% efficient is called their Förster radius (R_0) [9]. The efficiency of FRET swiftly drops to zero at distances larger than the Förster radius. The Förster radii are experimentally determined for each pair. For FAM and TAMRA pair $R_0 = 55 \text{ \AA}$ [10].

In the nanoblinker FRET Oligo FAM and TAMRA are at the distance of $23.8 \text{ \AA}$ [7]. Therefore, FRET efficiency (E_{FRET}) in this case is:

$$E_{\text{FRET}} = 1 / [1 + (r/R_0)^6] = 1 / 1.006565769 = 0.993477$$

In other words, FRET efficiency between FAM and TAMRA at this distance is 99.35%. Therefore in the uncleaved oligo the acceptor (TAMRA—red) is radiative, whereas the donor (FAM—green) is completely quenched. We investigated the nanoblinker FRET system in more detail elsewhere [7].

2. We recommend testing the nanoblinker system before performing any experiments with cells. Such controls can confirm the assay sensitivity. For these perform detection by using the exogenous 5'OH blunt-ended DNA in solution.

For the tests assemble the nanoblinker FRET probes in 100 mM Tris–HCl, pH 7.4 by combining 10 pmol (2 pmol/μL final concentration) FRET Oligo and 10 pmol (2 pmol/μL final concentration) vaccinia topoisomerase I (TOPO). To the reaction mix add 100 pmol of Test Oligo (20 pmol/μL final concentration) in 100 mM Tris–HCl, pH 7.4. Incubate the reaction for 1 min at room temperature before measurements. Scan samples using a spectrofluorometer at 488 nm excitation, or measure fluorescence of the donor (FAM) at 488 nm excitation and 525 nm emission wavelengths. The increase in 525 nm fluorescence indicates a positive reaction (see **Note 9** for more options).

Occasionally additional controls might be needed to exclude possible contamination of vaccinia topoisomerase preparations with nucleases. In such cases, the pretreatment of control sections with TOPO for 2 h at 37 °C is recommended followed by detection of DNA breaks.

3. The sensitivity of the assay depends on the biological model used, the labeling conditions, and the equipment for signal registration. In the biochemical tests using a Tecan Safire 2 scanning spectrofluorometer, the nanoblinker assay dependably detected 100 pmol of 5'OH DNA ends, represented by the Test Oligo (20 pmol/mL final concentration), after 30 s of incubation. In cell culture conditions after 3 min of incubation the assay was capable of detecting phagocytosis and reliably distinguished between apoptotic and necrotic cell engulfment based on the intensity of phagocytic activity of 20 phagocytes digesting 200 apoptotic or necrotic cells [7]. When used with fluorescence microscopy as in situ probes, the nanoblinders were applied to the cells grown on glass chamber slides. In these conditions the assay after 15 min of incubation fluorescently tagged individual phagolysosomes in macrophages digesting apoptotic and necrotic cells and enabled the subcellular level of analysis [7].
4. Active vaccinia topoisomerase I, which works well with the described assay, can be purchased from Millipore, sold as a part of the ApopTag® ISOL Dual Fluorescence Apoptosis Detection Kit. In most experiments we used the highly concentrated preparation of this enzyme obtained from Vivid Technologies (Houston, TX).
5. Cell lysis buffer is a proprietary phosphate-based buffer with pH ~ 7 supplied as part of the APO HTS 3/7 Caspase Detection kit from Cell Technology. Other approaches can be used to disrupt cells instead of this buffer, such as hypo-osmotic shock or freezing-thawing of cells. Prior to the application these approaches should be experimentally tested with the specific cell samples that will be investigated by the nanoblinker probes.
6. APO HTS 3/7 Caspase Detection kit from Cell Technology is the homogenous fluorimetric assay for active caspase 3/7, using $\lambda_{\text{excitation}} = 488 \text{ nm}$, $\lambda_{\text{emission}} = 525 \text{ nm}$.
7. As negative controls for the 18 h-long phagocytic reaction use cells without 5'OH DNA breaks, such as normal macrophages, apoptotic U87 cells, and necrotic

U87 cells. All negative controls cells (apoptotic, necrotic, and normal) should be incubated at 37 C for 18 h in parallel with the other (phagocytic) series of the experiment.

8. Strict 3 min incubation time was verified as sufficient for the sensitive measurement of specific DNA breaks in cultured cells [7]. However, the results also confirmed that the detection capability of the nanoblinker system was maintained for at least 15 min post assembly [7]. Therefore, it is highly likely that a different incubation interval within this time range can be used, if needed, but should be experimentally tested beforehand. In any case, the same exact incubation time should be used for all series.

9. FRET cessation in the TOPO probe signals detection of DNA breaks. Therefore, both the peak fluorescence of the donor at 525 nm and the ratio of donor and acceptor emissions at the excitation wavelength of the donor ($E_{D/A} = E_{D\ 525\ nm}/E_{A\ 580\ nm}$) can be used for the assessment of samples [7, 11, 12].

However, the use of the $E_{D/A}$ ratio provides a more sensitive measure because the disruption of FRET simultaneously increases the donor emission and diminishes the signal from the acceptor. Combined these two effects drive the $E_{D/A}$ ratio up [11, 12].

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Fig. 1.

Nanoblinker FRET assay for detection of DNase II breaks in cell suspensions. In the dual hairpin FRET Oligo the *green* DONOR fluorescence is suppressed due to FRET-based transfer of energy to the *red* ACCEPTOR (FAM and TAMRA fluorophores in the actual probe). Vaccinia topoisomerase I (TOPO) binds to the FRET Oligo and cleaves it in the center. Two hairpins perpetually ligate back and then recleave, producing alternating DONOR–ACCEPTOR fluorescence. In this equilibrium the ligated state is predominant and the main fluorescence is from the ACCEPTOR [7]. When DNase II breaks are present, the ACCEPTOR part ligates to them instead. This permanently stops FRET, eliminates *red* ACCEPTOR fluorescence, and activates *green* DONOR fluorophore in its place. The *color change* indicates detection

Fig. 2. Nanoblinker FRET probes detect 5' OH DNA ends. Emission spectra of cycling nanoblinkers before and after the addition of 5' OH DNA ends (Test Oligo). Before the reaction the emission maxima correspond to fluorescence of both DONOR (FAM—525 nm) and ACCEPTOR (TAMRA—580 nm). The detection reaction increases fluorescence of DONOR and decreases that of ACCEPTOR. $\lambda_{\text{Excitation}}=488$ nm. (see **Note 2** for full test details)