Supplementary Data

Anchored linear oligonucleotides: The effective tool for the real-time measurement of uracil DNA glycosylase activity

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1. Supplementary Methods

1.1 Synthesis of some used oligonucleotides

Some of the used oligonucleotides (ONs) were synthesized in Dr. Rosenberg IOCB laboratory as follows: single labelled ONs were synthesized in 0.5 µmol scale on BHQ1-modified LCAA CPG solid support (Glen Research) by standard phosphoramidite chemistry, using commercially available, protected 2'-deoxynucleoside-3'-phosphoramidites (ChemGenes). The synthesized ONs were purified by HPLC (Alliance, Waters) on a DNAPac anion exchanger column (10 × 250 mm; Dionex) using a linear gradient of sodium chloride (20 → 400 mM) in aqueous 20 mM sodium acetate containing 10% acetonitrile. Purified ONs were desalted on Luna C18(2) (10 µm; Phenomenex) using a thick-wall glass micro column (400 µl; IOCB mechanical workshops). The elution by a short linear gradient (0→100%; 20 min) of acetonitrile-methanol mixture (50-50) in water provided desalted ONs which were lyophilised and subjected to MALDI TOF to confirm correct mass.

1.2 M-sensor contains approximately 5 million probes

According to the manufacturer, the binding capacity of the magnetic particles for biotinylated oligonucleotides is 2.5 nmol/mg. In the case of 0.5 µg of magnetic particles per one well, the absolute amount of the bound oligonucleotide per well should correspond to ca 1.25 pmol of the probe. Measurement of the known, serially diluted concentrations of the biotinylated anchoring oligonucleotide B_{30FAM} showed that the real binding capacity in the used conditions was lower and corresponds to ca 0.6 pmol of the probe per 0.5 µg of magnetic particles. Due to the number of particles in the analysed sample (ca 75,140 particles in 0.5 µg), one such particle contained ca 4.8×10^6 of the probe on average.

1.3 Optimisation of the composition of the buffer for the measurement of glycosylase activity

Buffers with NaCl concentration ranging from 5.09 mM to 200 mM or $MgCl_2$ or $CaCl_2$ both ranging from 0.41 mM to 16 mM (dilution factor = 2) were tested with respect to the probe stability. In these experiments, one volume of m-sensors (with $B_{30FAM}C_{XQ}$ probe, see the table 1 in the manuscript) was added to 100 volumes of buffer containing 10 mM Tris-HCl, pH = 7.4, 1 mM EDTA and 200 mM NaCl or 16 mM $MgCl_2$ or 16 mM $CaCl_2$ and separated (the pellet was resuspended by pippeting). M-sensors were washed twice. After the last separation, two volumes of the above-mentioned buffers were added and the pellet was resuspended. Then, 2 µl of m-sensor suspension was mixed with 198 µl of the tested buffers so that the final concentration of the magnetic particles was 5 µg/ml. After a 30-minute incubation, 50 µl of the samples per well was added to the black 384-well plate and the fluorescent signal of 6-FAM was measured.
Next, the buffers containing 10 mM Tris-HCl, pH = 7.4, 1 mM EDTA and alternatively 50, 100, 150 mM NaCl or alternatively 1, 2, 4, 8 mM $CaCl_2$ or $MgCl_2$ were tested with respect to their impact on bacterial UNG activity or uracil glycosylase activity in nuclear lysates. Then, 50 µl of the washed m-sensors and 50 µl of bacterial UNG or nuclear lysate were added per well of the black 384-well plate and the fluorescent signal of 6-FAM was measured.

1.4 SPR assay optimization

The response of the used SPR sensor is expressed as a shift in the wavelength of SPR resonance and is directly proportional to the mass of biomolecules attached to the surface of the sensor. Using the calibration procedure described in [1], the surface density of both the immobilized probes and the subsequently attached molecules can be determined. For an SPR resonance of around 750 nm, the shift of 1 nm in the SPR wavelength represents a change in the protein surface coverage of 17 ng/cm^2 [1].
The efficiency of oligonucleotide hybridization (HE) is defined as the ratio of the formed duplexes to immobilized anchoring oligonucleotide probes, and was calculated as $HE = SR_{ON} \times M_{probe}/(SR_{probe} \times M_{ON})$, where M_{probe} and M_{ON} are the molecular weights of the biotinylated anchoring oligonucleotide probe and complementary second oligonucleotide, respectively; SR_{probe} is the equilibrium sensor response to the immobilization of the oligonucleotide probe, and SR_{ON} is the sensor response to the hybridization of complementary second oligonucleotide (figure 3 in manuscript). The SPR detection assay was optimised in terms of the surface density of the anchoring oligonucleotides (SDe) and oligonucleotide duplexes (DD) to yield a high UNG cleavage efficiency and a sufficiently high sensor response. Six different SDe and DD were tested with a model system of a $B_{30FAM}C_{UQ}$ probe ranging from 0.6 to 2.2 x10^{12} oligonucleotide/cm^2, and from 0.8 to 2.8 10^{12} duplexes/cm^2, respectively. For comparison, CE values normalized to the maximum value were used. As follows from the electronic supplementary material, figure S4, the optimum conditions for UNG activity were provided at SDe of 2.0 x 10^{12} anchoring oligonucleotide/cm^2 and DD of 1.3 x 10^{12} duplexes/cm^2 (corresponding to 65% HE), respectively.

1.5 Preparation of cell lysates using Triton X-100

The cell lysates prepared using Triton X-100 were prepared similarly as the cell lysates prepared by homogenisation with the following changes: 1.8 ml of buffer B was added to cells instead of 2 ml. After a 20-minute incubation on ice, 0.2 ml of ice-cold buffer D (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% Triton X-100 and protease inhibitors) was added, the solution was mixed by pipetting and incubated on ice for 5 min. The cells were scratched using the cell scraper, transferred into the ice-cold 2 ml tube and centrifuged 10 min. at 12,000 × g, 4°C. Both fractions were either used or aliquoted and stored at -80°C.

1.6 Microscopy evaluation of m-sensors

In experiments focused on the measurement of the fluorescence signal by fluorescence microscopy, all the images were acquired using an Olympus IX83 microscope (UPLFLN 2PH objective 10×, NA 0.3 or LUCPLFLN PH objective 20×, NA 0.45) equipped with a Zyla camera (Andor) with a resolution of 1,024 × 1,024 pixels using acquisition software (CellSense Dimension, Olympus) [2]. The glycosylase activity of bacterial UNG on the m-sensors were acquired every 2 minutes in 15 cycles. The obtained data were analysed using CellProfiler [3, 4] and Microsoft Excel software and the final graphs were plotted in GraphPad Prism 6 [2].

References:

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2. Ligasova A, Vydrzalova M, Burianova R, Bruckova L, Vecerova R, Janost'akova A et al. A New Sensitive Method for the Detection of Mycoplasmas Using Fluorescence Microscopy. Cells-Basel. 2019;8(12). doi:10.3390/cells8121510
3. Carpenter, AE, Jones, TR, Lamprecht, MR, Clarke, C, Kang, IH, Friman, O, Guertin, DA, Chang, JH, Lindquist, RA, Moffat, J, et al. 2006 CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 7, R100. (10.1186/gb-2006-7-10-r100)
4. Kamentsky, L, Jones, TR, Fraser, A, Bray, MA, Logan, DJ, Madden, KL, Ljosa, V, Rueden, C, Eliceiri, KW, Carpenter, AE. 2011 Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. Bioinformatics. 27, 1179-1180. (10.1093/bioinformatics/btr095)

2. Supplementary Results

2.1 Impact of the different m-sensor concentrations on the signal

The feasibility of the assay was verified over a wide range of m-sensor concentrations and it was demonstrated that the rate of the signal growth after bacterial UNG addition increased linearly with the m-sensor concentration (electronic supplementary material, figure S2). To compensate for the effect of procedural imperfections on the method reproducibility, the m-sensors labelled with E_{Cy5} probe were used as an internal standard. This step allowed a significant decrease of the impact of the various amounts of the added m-sensors. While the speed of the signal growth of 6-FAM increased linearly in the whole range of measurement, the speed of the signal growth calculated from 6-FAM and Cy5 ratio changed distinctly less (electronic supplementary material, figure S2). The intra-assay reproducibility was estimated from the signal collected from fifty different wells with Cy5 internal standard and single concentration of m-sensors and was shown to be higher than 97%. Therefore, it is possible to exclude the correction of the amount of m-sensors in most experiments. On the other hand, the simultaneous labelling of m-sensors with another fluorochrome is simple and provides an easy way to control the quality of data and also the possibility of eventual correction.
The dependence of the speed of the signal growth on the concentration of m-sensors in nuclear lysates was non-linear and levelled off at the m-sensors' amount above 0.25 µg per well (electronic supplementary material, figure S3, compare with the left figure of electronic supplementary material, figure S2 for bacterial UNG). Although it was not possible to eliminate the impact of the different m-sensor concentrations using an independent marker in the case of cell lysates, it is obvious that the inaccuracy caused by pipetting should be lower in experiments with cell lysates comparing to the bacterial UNG if more than 0.25 µg of m-sensors is applied per well. As the error introduced by pipetting was also low (less than 3%, see above) the correction on the amount of m-sensors was left out in the case of nuclear lysates.

2.2 Microscopy evaluation of m-sensors

Simultaneously with the analysis of m-sensors by plate reader, the use of fluorescence microscopy was tested. We used m-sensors with $B_{30FAM}C_{UQ}$ or $B_{30FAM}C_{TQ}$ probes and bacterial UNG in this experiment (Electronic Supplementary Material Video S1 and Video S2, Electronic Supplementary Material Fig. S5). The results obtained from fluorescence microscopy were similar to the results from the plate reader (Fig. 1b in the manuscript). The disadvantage of the microscopic system is the slower acquisition and evaluation of samples. Although this disadvantage can be partially eliminated by the use of automated microscopic stations and suitable software, microscopy stations are usually much more expensive than evaluation using a plate reader. On the other hand, the microscopic system allows study of the individual particles and, therefore, study of the dynamics of changes in various parts of the tested sample. From this point of view, the use of m-sensors could be an interesting possibility for addressing the situation in individual cells and their compartments. For this purpose, however, it will be necessary to use the particles with smaller diameter and also a system allowing their fast transfer into cells.

3. Supplementary Figures

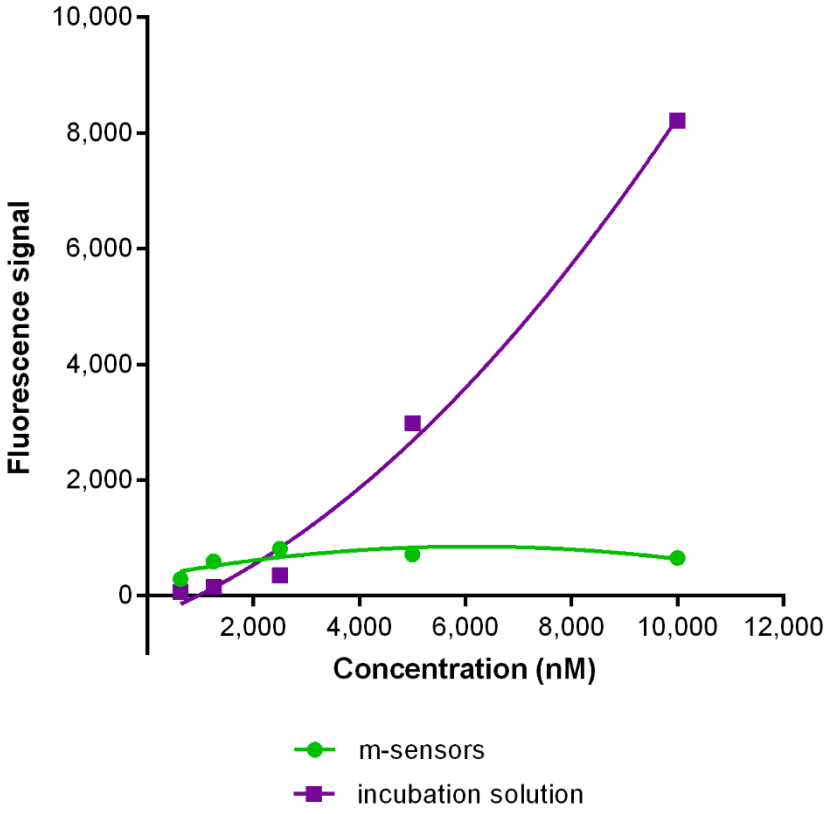

Figure S1. Dependence of the signal of m-sensors on the concentration of the oligonucleotide
Dependence of the signal of m-sensors with a biotinylated anchoring oligonucleotide B_{30FAM} and the signal of the free biotinylated anchoring oligonucleotide B_{30FAM} remaining in the incubation solution on the concentration of the oligonucleotide is shown. The magnetic particles (1 mg/ml) were incubated with various concentrations of the biotinylated anchoring oligonucleotide B_{30FAM}. After one hour, the m-sensors were separated using the UniTrap magnetic separator. The incubation solution was transferred into the new tube. The m-sensors were washed and re-suspended in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween and 0.02% sodium azide (TBT buffer). The fluorescent signal of 6-FAM was measured both in the remaining incubation solution and in the solution of m-sensors in the TBT buffer.

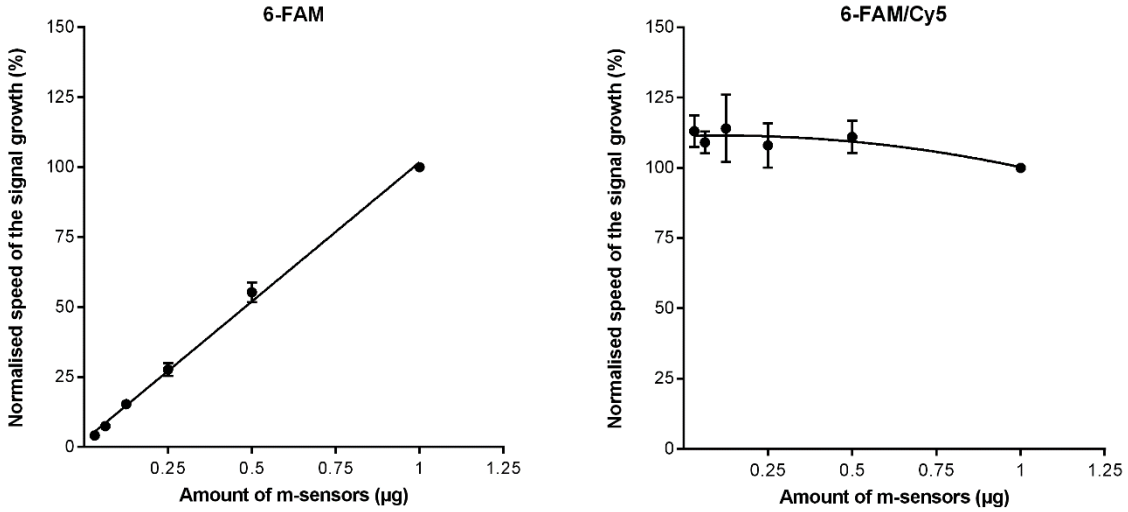

Figure S2. Dependence of the speed of the signal growth on the amount of m-sensors and impact of 6-FAM/Cy5 ratio

Dependence of the speed of the signal growth of m-sensors in the solution of bacterial glycosylase on the amount of m-sensors containing the $B_{30FAM}C_{UQ}$ probe and impact of the division of the 6-FAM signal by Cy5 signal is shown. The solution of bacterial UNG (40 mU/ml) was added to the black 384-well plate with various amounts of m-sensors (alternatively 1, 0.5, 0.25, 0.125, 0.0625 or 0.03125 µg). The samples were incubated at 25°C. During incubation, the fluorescence signal of 6-FAM and Cy5 was acquired every two minutes (10 cycles). The 6-FAM signal was (right) or was not (left) divided by the Cy5 signal. The speed of the signal growth was determined as a value of the first derivation of the function at the beginning of the measurement. The speed of the signal growth was normalised to the speed of the signal growth in samples with the highest concentration of m-sensors equal to 100%. The data are shown as the mean ± SD.

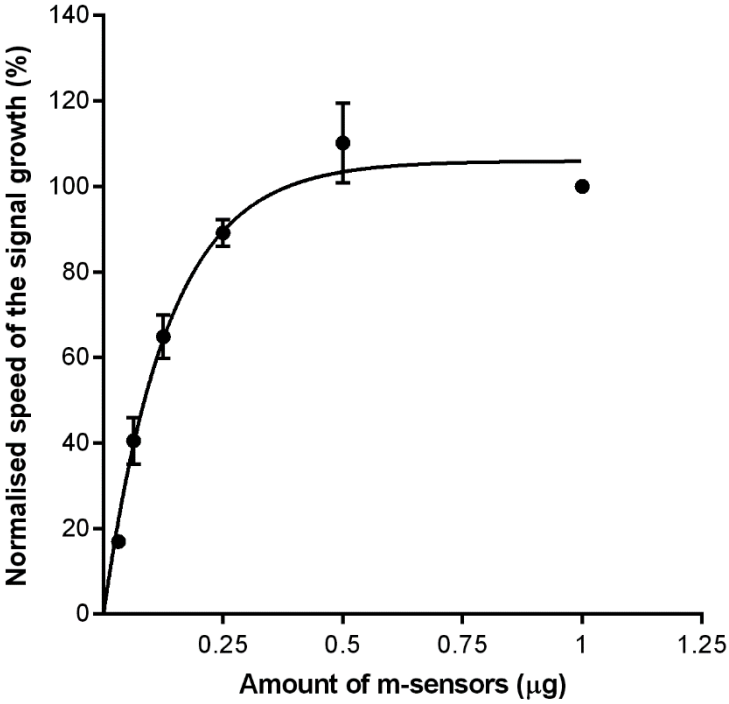

Figure S3. Dependence of the speed of the signal growth on the m-sensor amount in nuclear lysate of HeLa cells

Dependence of the speed of the signal growth on the m-sensor amount in the solution of nuclear lysate of HeLa cells is shown. The solution of nuclear lysate from HeLa cells (2 μg/ml of the overall protein) was added to the black 384-well plate with various amounts of prepared m-sensors (alternatively 1, 0.5, 0.25, 0.125, 0.0625 or 0.03125 μg) and samples were incubated at 25°C. During incubation, the fluorescence signal of 6-FAM was measured every two minutes (10 cycles). The speed of the signal growth was determined as a value of the first derivation of the function at the beginning of the measurement. The speed of the signal growth was normalised to the speed of the signal growth in samples with the highest concentration of m-sensors equal to 100%. The data are shown as the mean ± SD.

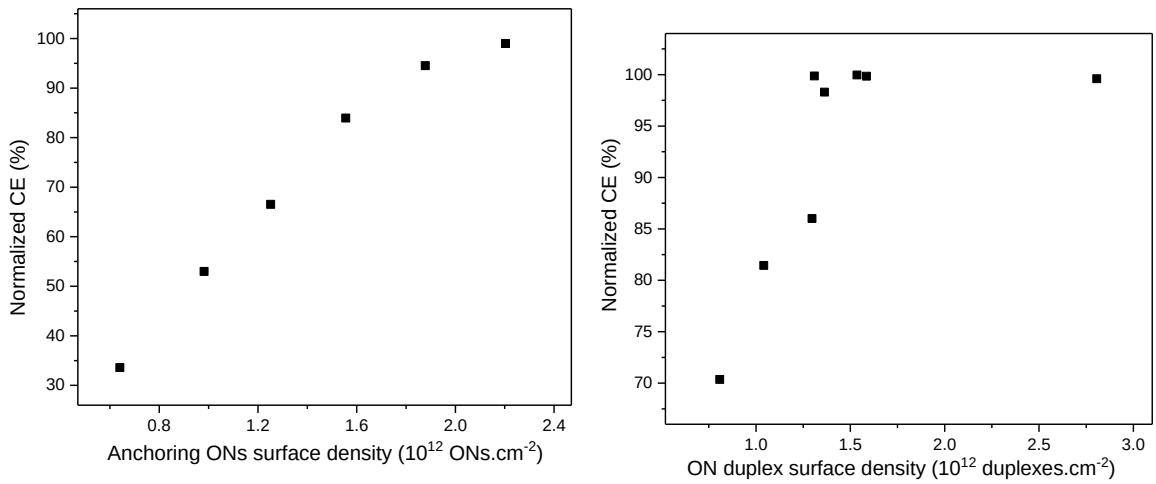

Figure S4. Dependence of the normalized CE on the surface density of anchoring oligonucleotides and oligonucleotide duplexes

Dependence of the normalized CE on the surface density of anchoring oligonucleotides (ONs, left) and oligonucleotide duplexes (ON duplex, right) is shown.

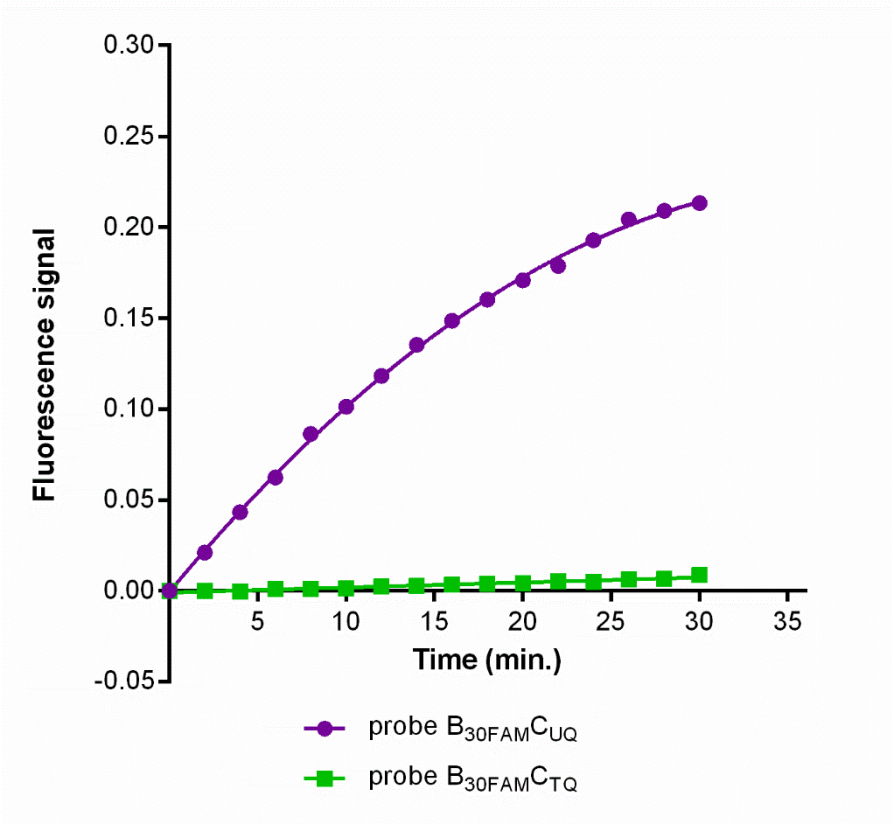

Figure S5. Analysis of glycosylase activity using fluorescence microscopy
Comparison of the growth of the signal in m-sensors with $B_{30FAM}C_{UQ}$ and $B_{30FAM}C_{TQ}$ probes in the presence of bacterial UNG by fluorescence microscopy is shown. During incubation, the fluorescence signal of 6-FAM was acquired every two minutes.

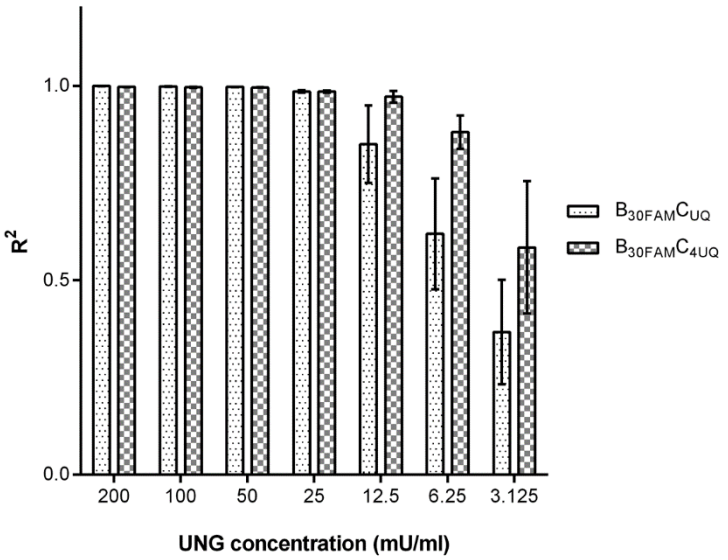

Figure S6. Dependence of the coefficient of the determination R^2 on UNG concentration and number of uracils.

The dependence of the coefficient of determination R^2 on UNG activity and the number of uracils calculated from the data used for Figure 5b. For the graph construction, we used the values of R^2 obtained from the linear regression. The data are shown as the mean ± SD.

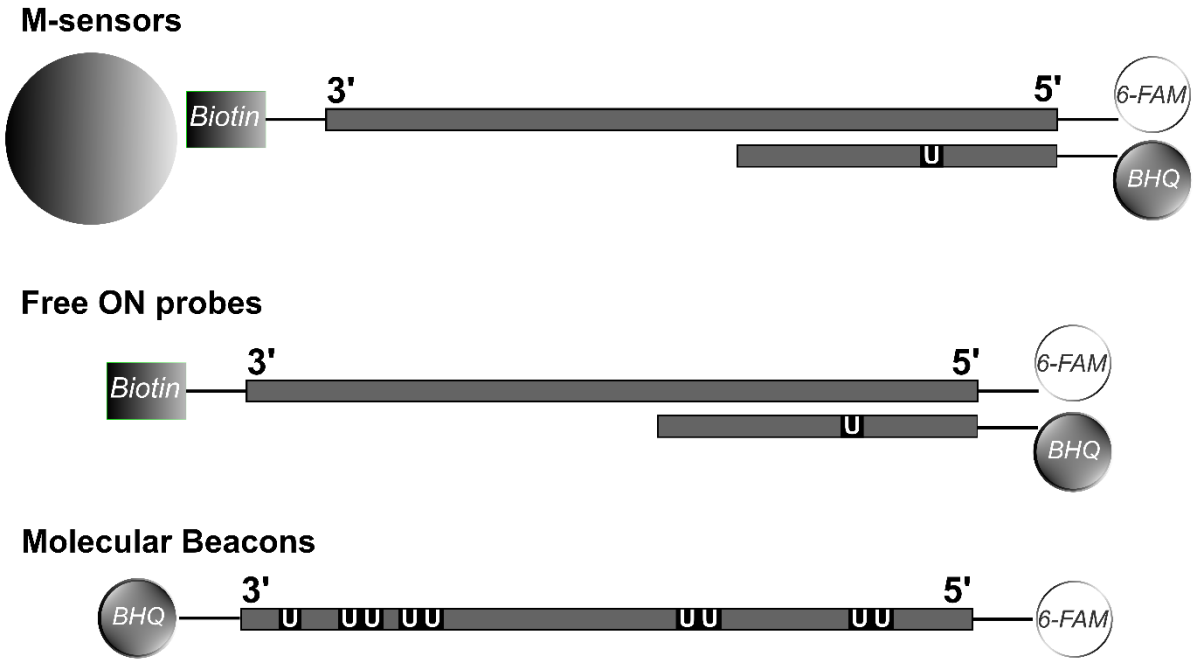

Figure S7. Scheme of the m-sensors, free probes and molecular beacons
Scheme of the m-sensors, free probes and molecular beacons used for the comparison of their use for the glycosylase activity analysis.

<u>The sequences of oligonucleotide (ON) duplex used in m-sensors and probes:</u>

1, Control ON duplex

3´- CGC CTA CAG CAG CGC CAA ATT CTT AAG TGC-5´
5´-TT TAA GAA TTC ACG-3´

2, ON duplex with uracil

3´- CGC CTA CAG CAG CGC CAA ATT CTT AAG TGC-5´
5´-TT TAA GAA UTC ACG-3´

<u>The sequence of the molecular beacons:</u>

1, Control molecular beacon

3´-CGT GAA TTC TTA AAG CTG TAC CGC ACT TAA GAA TTC ACG-5´

2, Molecular beacon with uracils

3´-CGU GAA UUC UUA AAG CTG TAC CGC ACU UAA GAA UUC ACG-5´

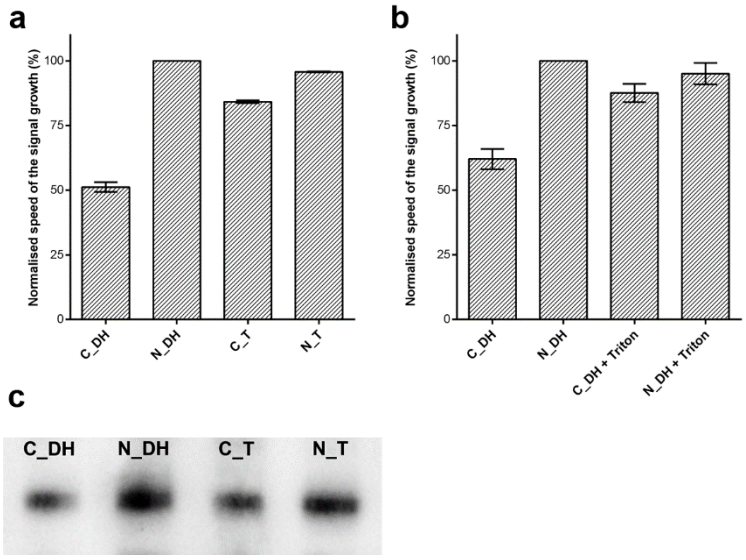

Figure S8. Impact of the preparation of cellular lysates on the glycosylase activity and UNG content

a) The impact of the preparation of cellular lysates on the glycosylase activity was measured in cytoplasmic (C) and nuclear (N) lysates prepared either by Dounce homogeniser (DH) or Triton X-100 (T). The prepared lysates were incubated with m-sensors with $B_{30FAM}C_{UQ}$ or $B_{30FAM}C_{TQ}$ probes. The speed of the signal growth was normalised to the speed of the signal growth in nuclear lysates prepared by Dounce homogeniser equal to 100%. The data are shown as the mean ± SD.

b) The impact of the addition of Triton X-100 to cellular lysates prepared by Dounce homogeniser on the glycosylase activity. The glycosylase activity was measured in cytoplasmic (C) and nuclear (N) lysates prepared either exclusively by Dounce homogeniser (C_DH and N_DH) or after the addition of Triton X-100 to the lysates prepared by Dounce homogeniser (C_DH + Triton and N_DH + Triton). The prepared lysates were incubated with m-sensors with $B_{30FAM}C_{UQ}$ or $B_{30FAM}C_{TQ}$ probes. The speed of the signal growth was normalised to the speed of the signal growth in nuclear lysates prepared by Dounce homogeniser equal to 100%. The data are shown as the mean ± SD.

c) The analysis of the content of UNG2 in nuclear (N) and cytoplasmic (C) lysates prepared either by Dounce homogeniser (DH) or by Triton X-100 (T) using Western blot is shown.

4. Supplementary Tables

Table S1. The measured signal of m-sensors with uracil probes in the solutions containing the lowest tested concentration of ions

the lowest concentration of ions	measured signal
5.09 mM NaCl	162.71 ± 1.51
0.41 mM $CaCl_2$	148.31 ± 5.32
0.41 mM $MgCl_2$	134.75 ± 5.48

5. Supplementary Videos

Video S1. Example of measurement of the signal by fluorescence microscopy

M-sensors with $B_{30FAM}C_{UQ}$ probe were added to the black glass-bottom 96-well plate and left on the neodymium magnet to let the magnetic particles settle. Then, a solution of bacterial UNG was added and the signal was measured every 2 minutes for 15 cycles.

Video S2. Example of measurement of the signal by fluorescence microscopy

M-sensors with $B_{30FAM}C_{TQ}$ probe were added to the black glass-bottom 96-well plate and left on the neodymium magnet to let the magnetic particles settle. Then, a solution of bacterial UNG was added and the signal was measured every 2 minutes for 15 cycles.