Real-Time Tracking of Yb$^{3+}$, Tm$^{3+}$ Doped NaYF$_4$ Nanoparticles in Living Cancer Cells

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The aim of the study was to demonstrate the possibility of real-time tracking of polyethylenimine-coated NaYF$_4$:Yb,Tm upconversion nanoparticles (UCNPs) in living cancer cells using wide-field microscopy technique.

Materials and Methods. Human breast adenocarcinoma SK-BR-3 cells and Yb$^{3+}$, Tm$^{3+}$ doped NaYF$_4$ nanoparticles with anti-Stokes photoluminescence were used in the study. The nanoparticles were visualized using wide-field microscope with excitation at 975 nm and signal detection in 420–842 spectral range. The analysis of the displacement of UCNPs was performed by fitting the point spread function of the photoluminescent spots corresponding to UCNP location by the Gaussian function, and calculation of mean square displacement.

Results. UCNPs were rapidly internalized by SK-BR-3 cells and retained in the cells for at least 12 h. Two types of the particles motion were registered: (i) isotropic random spatial fluctuations with relatively small amplitudes and low rate of displacement, and (ii) flick and directional movements with rates up to 1.2 µm/s and total displacement up to tens of microns. The registered types of motion can be attributed to diffusion in local area and intracellular transport of nanoparticles encapsulated in vesicles, respectively.

Conclusion. The demonstrated tracking of UCNPs in human breast adenocarcinoma cells showed that Yb$^{3+}$, Tm$^{3+}$ doped NaYF$_4$ nanoparticles are an advanced agent for dynamic studies of intracellular processes. The implemented scheme for UCNPs tracking provides long-term observation with preservation of cell viability for at least several hours. In total, almost complete absence of cell autofluorescence and UCNPs photobleaching, low invasiveness, fast rate of image acquisition allow us to consider the proposed approach as useful for a variety of tasks in biomedical research.

Key words: upconversion nanoparticles; single-particle tracking; wide-field microscopy.


Introduction

The study of molecular transport, underlying biological processes, is one of the actively developing fields of science. The modern technical equipment for visualization and tracking of single molecules labeled with fluorescent probes allows real-time detection, identification, and tracking of individual molecules in biological systems [1]. The specificity of tracking in biological systems causes improved criteria for fluorescent dyes. In particular, it is

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The possibility was shown to visualize intracellular common use due to the high efficiency of upconversion excitation. Photoluminescence (PL) intensity during prolonged properties of the dye throughout the experiment. Thus, necessary to ensure the consistency of the photophysical biophotonics in cancer research erbium or thulium ions (NaYF4:Yb,Er/Tm) has got the most UCNPs with a large selection of doping elements [16, 18]. Varieties of inorganic matrices can serve as a base for Upconversion nanoparticles (UCNPs) have recently emerged as a new class of inorganic photoluminescent nanoparticles that can successfully replace quantum dots [10]. UCNPs effectively convert the energy of absorbed infrared photons (~975 nm) into visible and near-infrared radiation. Unique photophysical properties of UCNPs, such as programmable spectra and narrow PL peaks, significant depth of optical detection in vivo [11], resistance to photobleaching [12, 13] and low cytotoxicity [14, 15], have provided their wide use in various types of biomedical research [16, 17]. Variety of inorganic matrices can serve as a base for UCNPs with a large selection of doping elements [16, 18]. However, the fluoride matrix co-doped with ytterbium and erbium or thulium ions (NaYF4:Yb,Er/Tm) has got the most common use due to the high efficiency of upconversion (energy conversion). Remarkable photostability of UCNPs and the noninvasiveness of near-infrared excitation provide long-term visualization of processes of interest. The possibility was shown to visualize intracellular migration of NaYF4 nanoparticles co-doped with Yb3+ and Er3+ for 6 h of continuous image acquisition [19]. Also, the use of similar UCNPs for rapid and background-free 3D visualization of dynamic processes in living cells was demonstrated [20]. Excitation of UCNPs in the infrared range provides an absence of cell autofluorescence, which almost completely eliminates the background signal out of focus and, therefore, greatly simplifies the determination of the 3D coordinates of UCNPs localization.

The aim of the study was to demonstrate the possibility of real-time tracking of NaYF4:Yb,Tm upconversion nanoparticles coated with polyethyleneimine in living cancer cells using wide-field microscopy technique. Materials and Methods Nanoparticles. Hexagonal NaYF4 nanocrystals containing 8 mol% Tm3+, co-doped with 20 mol% Yb3+ [21] were used in the work. To achieve colloidal stability of NaYF4:Yb,Tm UCNPs in aqueous solutions they were surface coated with polyethyleneimine (UCNP-PEI) [14]. The transmission electron microscopy images were obtained using CM10 microscope (Philips, The Netherlands) with an accelerating voltage of 100 kV and a resolution of 5.0 Å for a point and 3.4 Å for a line.

Cells. The cell line SK-BR-3 (human breast adenocarcinoma, ATCC number HTB-30) was cultured in the McCoy’s 5A growth medium with 10% (v/v) fetal calf serum (HyClone, USA) and 2 mM L-glutamine in 5% CO2 at 37°C. For passaging the cells were carefully detached using Versen solution (PanEco, Russia).

Tracking of UCNPs in SK-BR-3 cells. SK-BR-3 cells were seeded in 96-well plates (BD Falcon, USA) at the density of 1·10⁴ cells per well and cultured for 24 h at 37°C in 5% CO2. The growth medium was then replaced with PBS containing 5 μg/ml UCNPs-PEI, and the cells were incubated for 2 h. After that, the UCNPs-PEI solution was replaced with fresh growth medium, and the cells were incubated overnight. Tracking of UCNPs-PEI in SK-BR-3 cells was carried out using the home-built setup created on the basis of the Axio Observer A1 microscope (Carl Zeiss, Germany). The scheme of the setup is shown in Figure 1.

The exciting radiation is generated by a laser diode LDD-10 (Semiconductor devices, Russia) at 975 nm. Then, the laser diode emission is assembled into a multimode optical fiber (200 μm diameter) connected to a collimator F280SMA-980 (Thorlabs, USA), which forms a Gaussian beam with a diameter of 2 mm. Further, a uniform illumination of the sample is created using a lens focusing on the back focal plane of an immersion oil objective C Plan Apochromat 63× NA1.4 Oil (Carl Zeiss, Germany). On the way to the sample, the excitation radiation is spectrally filtered by a laser clean-up filter LD01-975/10 (Semrock, USA), after that the radiation is reflected from the dichroic mirror Z1064rdc-sp (Chroma, USA) and focused on the sample using the objective. The PL from the sample passes through the dichroic mirror and two successively set edge short-pass filters FF01-842/SP (Semrock, USA) and focused on the back focal plane of an immersion oil objective C Plan Apochromat 63× NA1.4 Oil (Carl Zeiss, Germany). On the way to the sample, the excitation radiation is spectrally filtered by a laser clean-up filter LD01-975/10 (Semrock, USA), after that the radiation is reflected from the dichroic mirror Z1064rdc-sp (Chroma, USA) and focused on the sample using the objective. The PL from the sample passes through the objective, dichroic mirror, and two successively set edge short-pass filters FF01-842/SP (Semrock, USA), which cut off laser radiation and transmit the spectral range of the PL registration (420–842 nm). The image is captured through the side port of the microscope to the iXon3 897 EMCCD camera (Andor, UK).

The excitation of the samples was carried out in a continuous mode at 975 nm with a radiation intensity of 950 W/cm² and an image acquisition time of up to 30 min. Registration of epi-luminescence images was performed in a mode of continuous image acquisition (frame transfer kinetics) with a frequency of ~5 Hz and an exposure of each frame of 200 ms. Using a red LED, the sample was illuminated every 5 frames to obtain brightfield images...
in which the location and morphology of the cells were
determined. Synchronization of LED start was carried
out by means of the pulse generator AKIP-3413/1 (Prist,
Russia), which received a control signal from the iXon3
897 camera (Andor, UK). All images were acquired at
room temperature.

Analysis of trajectories of UCNP-PEI. The PL
signal of nanoparticles with a size below the diffraction
limit is well described by the normal distribution.
Therefore, to determine the coordinates of the UCNP-
PEI location in cells, we fitted the diffraction spots
corresponding to UCNP-PEI by the two-dimensional
Gaussian function (G) [22]:

\begin{equation}
G(x,y) = I_0 \exp \left[ - \frac{(x-x_0)^2}{2s_x^2} - \frac{(y-y_0)^2}{2s_y^2} \right],
\end{equation}

where \(x_0, y_0\) is the center of the diffraction spot, \(2\sqrt{\ln 2} \cdot s_{x,y}\)
is its full width at half maximum along the axis \(x\) and \(y\),
respectively and \(I_0\) is the peak intensity.

To determine the transport speed and the type
of motion of the marked objects, the mean square
displacement (MSD) was calculated for each time point
of the UCNP-PEI trajectory:

\begin{equation}
\text{MSD}(n\tau) = \frac{1}{N-n} \sum_{i=1}^{N-n} [(x_i[(i+n)\tau] - x_i(i\tau))^2 +
(y_i[(i+n)\tau] - y_i(i\tau))^2],
\end{equation}

where \(\tau\) is the acquisition time, \(N\) is the total number of
frames, and \(n\) is the frame number in sequence.

The plot MSD(n\tau) has characteristic features for each
type of particle motion. The linear dependence of MSD on
time with a slope of \(4D\), where \(D\) is diffusion coefficient,
corresponds to an isotropic random motion. If there is an
additional directional motion with a velocity \(\nu\), MSD(n\tau) is
expressed as:

\begin{equation}
\text{MSD}(n\tau) = 4Dn\tau + (\nu n\tau)^2.
\end{equation}

In the case when the random motion of a particle is
bounded by the region \(L\), the graph MSD(n\tau) is described
by the following expression:

\begin{equation}
\text{MSD}(n\tau) = \frac{L^2}{3} \left[ 1 - \exp \left( \frac{12Dn\tau}{L^2} \right) \right].
\end{equation}

Results and Discussion

According to the transmission electron microscopy
data on the size distribution, NaYF₄:Yb,Tm UCNPs
were monodisperse with an average nanocrystal size of
28.1±2.2 nm (Figure 2). This type of Tm³⁺-doped UCNPs
is characterized by PL emission with several main peaks
in the visible (455 nm, 514 nm, and 660 nm) and near-
infrared (744 nm, 782 nm, and 802 nm) spectral regions
when excited at 980 nm [21].

Incubation of UCNP-PEI with SK-BR-3 cells for 2 h
was accompanied by receptor-independent endocytosis.
The internalized nanoparticles were retained in the
cell for a long time (at least 12 h). We visualized the
behavior of particles in the cell after this time period.
Figure 3 shows images of UCNP-PEI in SK-BR-3 cell
extracted from a video file recorded in real-time at a
rate of 5 frames per second. Taking into account the size of UCNPs, which is about ~30 nm, the most likely mechanism of the nanoparticles uptake is pinocytosis, leading to encapsulation of particles in vesicular organelles such as endosomes and their subsequent intracellular transport [23, 24]. The positive surface charge of the PEI-coated UCNPs, presumably, leads to the electrostatic interaction with negatively charged membrane phospholipids, such as phosphatidylserine and phosphatidyglycerol, and facilitates the first steps of the internalization.

The proposed scheme for recording luminescence causes a selective signal acquisition only from UCNP-PEI (Figure 3, middle row). When exciting UCNPs by a continuous wave laser diode at 975 nm, the autofluorescence signal is undetectable [20]. The blurred PL signal is caused by UCNPs located outside the focus at the time of exposure of the frame.
While visually analyzing the video files obtained, we noted that some particles undergo flick and directional movements. These movements usually last for several seconds and resume after short pauses, which is typical for the intracellular transport of nanoparticles encapsulated in vesicles [19, 25]. In particular, such behavior is demonstrated by the particle marked under number 1 in Figure 3. At the same time, some of the UCNP-PEI particles perform random spatial fluctuations with relatively small amplitudes (see the particle marked under number 2 in Figure 3).

To determine the location of particles 1 and 2, we have fitted the PL spots corresponding to these particles by the Gaussian function (1) on each frame of the time interval from 0 to 37.3 s. Figure 4 shows an example of determining the UCNP-PEI location.

The particle trajectories calculated in this way are shown in Figure 5. Particle 2 moves in a limited region with an area of less than 4 μm², while particle 1 is moved to more than 14 μm from its original location. The most likely mechanism for the particle 1 moving is active intracellular transport through microtubules or actin filaments [26].

Figure 6 shows the graphs of cumulative and relative displacements, depending on the time of observation of

Figure 4. Determination of the location of the vesicle containing the UCNP-PEI particles by fitting by the Gaussian function. The scale bar is 1 μm

Figure 5. Trajectories of the UCNP-PEI particles 1 and 2 in SK-BR-3 cell
On the right and left inserts, the red color indicates the pathway traversed in 37.3 s by the particles 1 and 2, respectively. The scale bar on the inserts is 1 μm

Figure 6. Cumulative and relative displacements of particles 1 and 2, depending on the time of the observation
the UCNP-PEI particles indicated in Figure 3. The velocity of motion of the two types of particles noted above calculated on the basis of cumulative displacement differs several times (about 2.5). In order of magnitude, the particle velocities determined in our work (0.4±1.2 μm/s) agree with the data of [19], in which the tracking of UCNPs in a cell was performed.

Another indicator shown in Figure 6 is the relative displacement (blue curves). In the case of direction motion, the displacement reached a significant value (Figure 6 (a)), whereas for random motion the displacement did not exceed 2 μm (Figure 6 (b)).

An important aspect that must be taken into account when carrying out long-term observations of living cells is the preservation of their viability throughout the experiment. In our study, the cells remained viable for 3 h. This opens possibilities for long-term monitoring of biological processes of interest in a cancer cell.

**Conclusion**

In this paper, we implemented a scheme for tracking of NaYF4:Yb,Tm nanoparticles coated with polyethylenimine in living cells using wide-field microscopy. The observed nonspecific tracking of UCNP-PEI in human breast adenocarcinoma cells SK-BR-3 showed that UCNPs are an advanced agent for dynamic studies of intracellular processes.

The key advantages of the proposed tracking scheme with the use of UCNP-PEI and wide-field microscopy are the almost complete absence of autofluorescence, which ensures a high selectivity of PL registration, and fast rate of image acquisition, which makes it possible to study fast intracellular processes.

Further improvement of the photophysical properties of UCNPs and the use of surface conjugation schemes will expand the use of UCNPs as a PL marker for monitoring intracellular processes.

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**Conflicts of interest.** The authors declare that there are no conflicts of interest regarding the publication of this paper.

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