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Biomedical applications of nanoflares: Targeted intracellular fluorescence probes

Karim Khanmohammadi Chenab, BSc[a], **Reza Eivazzadeh-Keihan, BSc**[a,1], **Ali Maleki, PhD**[a], **Paria Pashazadeh-Panahi, MSc**[b,c], **Michael R Hamblin, PhD**[d,e,f,*], and **Ahad Mokhtarzadeh, PhD**[c,g,**]

[a]Catalysts and Organic Synthesis Research Laboratory, Department of Chemistry, Iran University of Science and Technology, Tehran, Iran

[b]Department of Biochemistry and Biophysics, Metabolic Disorders Research Center, Gorgan Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Golestan Province, Iran

[c]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[d]Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, USA

[e]Department of Dermatology, Harvard Medical School, Boston, MA, USA

[f]Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, USA

[g]Department of Biotechnology, Higher Education Institute of Rab-Rashid, Tabriz, Iran

Abstract

Nanoflares are intracellular probes consisting of oligonucleotides immobilized on various nanoparticles that can recognize intracellular nucleic acids or other analytes, thus releasing a fluorescent reporter dye. Single-stranded DNA (ssDNA) complementary to mRNA for a target gene is constructed containing a 3′-thiol for binding to gold nanoparticles. The ssDNA "recognition sequence" is prehybridized to a shorter DNA complement containing a fluorescent dye that is quenched. The functionalized gold nanoparticles are easily taken up into cells. When the ssDNA recognizes its complementary target, the fluorescent dye is released inside the cells. Different intracellular targets can be detected by nanoflares, such as mRNAs coding for genes over-expressed in cancer (epithelial-mesenchymal transition, oncogenes, thymidine kinase, telomerase, etc.), intracellular levels of ATP, pH values and inorganic ions can also be measured. Advantages include high transfection efficiency, enzymatic stability, good optical properties,

*Correspondence to: M.R. Hamblin, Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, 02114, USA. hamblin@helix.mgh.harvard.edu, (M.R. Hamblin). **Correspondence to: A. Mokhtarzadeh, Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. ahad.mokhtarzadeh@gmail.com. (A. Mokhtarzadeh).
[1]Co-first authors.

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biocompatibility, high selectivity and specificity. Multiplexed assays and FRET-based systems have been designed.

Keywords

Nanoflares; Targeted intracellular fluorescence probes; Nucleic acid hybridization; Cancer cell detection; mRNA detection; ATP detection; Inorganic ion detection

Introduction

Imaging of intracellular processes has remained challenging over the last few decades. The study of intracellular processes has helped scientists to elucidate biological mechanisms, understand gene expression, and explore various enzymatic activities. Over many years, cancer researchers have gained a large body of knowledge about what makes cells become cancerous or malignant. Nevertheless, imaging of intracellular processes within living cells in real time has remained an unsolved challenge. Intracellular physicochemical parameters are difficult to measure without disturbing the cell. A living cell is a complicated system whose intricate workings are ultimately controlled by nucleic acids. Genetic mutations are the main reason for initiation of unwanted and unpredictable processes that result in cancer formation. Therefore, intracellular studies of nucleic acid expression, production and trafficking have attracted much attention. Fluorescent methods relying on external excitation techniques such as those based on Forster resonance energy transfer,[1] molecular beacons labeled with fluorescent molecules,[2] in situ hybridization and staining with fluorescent antibodies,[3] have all been investigated for detection and study of intracellular molecules. In the field of intracellular biomolecular detection, efficient transfection into cells, stability of reagents in the presence of cellular enzymes,[4] and fluorescent quenching[5] are the main challenges to be overcome. According to many reports, externally delivered fluorescence probes suffer from many unsolved problems, despite having many benefits. Externally delivered probes the advantages of low cost, better availability, and the possibility to be applied for intracellular imaging of a broad range of cells. One of the main disadvantages of these methods however, is an intrinsic limitation on the intensity of fluorescence, and difficulty to generalize the reagents to a variety of target biomolecules inside cells. Internalization of reagents such as antibodies and aptamers into cells is challenging. Moreover, supplementary reagents are often necessary. Unfortunately, these supplementary reagents do not usually have sufficient chemical stability against enzymatic degradation inside cells.[6] In addition transfection reagents, such as lipids[7] and dendrimers[8] can show harmful and toxic side effects. The uptake process of oligonucleotides (which are a critical component of nanoflares) into cells is a significant challenge limiting their use in intracellular imaging processes.

With the discovery of oligonucleotides immobilized onto nanomaterials, many investigators have explored their application to intracellular imaging.[9–12] According to recent reports, oligonucleotide-based nanoflares have many advantages that suggest they can play key roles in optical biosensors, for genetic analysis and bio-delivery systems. Generally, nanoflares are formed from nanoparticles with attached oligonucleotides as substrate and probe,

respectively. The intrinsic properties of immobilized oligonucleotide-based nanoflares may provide many advantages in imaging of different intracellular species such as DNA, RNA and so on. Different nanoparticulate substrates have been used to immobilize and quench the nanoflares. On one hand, noble metal (Au, Ag, Cu etc.) nanoparticles are good candidates as immobilizing substrates, and as optical quenchers of nanoflares because of their appropriate surface plasmon resonance properties.[13] On the other hand, oligonucleotides can possess a highly efficient transfection ability (without any supplementary reagents), have good stability against intracellular enzymatic degradation, and show high sensitivity to detect complementary RNA and DNA sequences. Although the optical properties of nanoflares can be affected by the composition of the cellular milieu, noble metal nanoparticle-based nanoflares exhibit distance-dependent optical properties, with efficient fluorescent emission, high sensitivity for RNA transcripts, and very weak background fluorescence when used for intracellular imaging.[14]

The understanding of the mechanism of fluorescence in nanoflares plays a key role in the design of optical sensors. So, as a brief introduction to the fluorescence spectroscopy of nanoflares we can mention emission, absorption wavelength and intensity. Typically, many environmental and molecular interactions can reduce the fluorescence intensity, which are called quenching effects. Formation of molecular complexes between fluorophore and other ground state molecules leading to energy transfer between the species leads to quenching. The optical properties of different nanomaterials mean that they can be used both as quenchers or probes in fluorescent-based imaging systems. The nanomaterials that can be used in nanoflares, must have specific optical properties. The crystal phase, size, and band gap of nanoparticles define the absorption and emission properties. Four different types of nanoparticles have been used as substrates and/or probes in nanoflares. These nanoparticles are (a) gold nanoparticles and nanoclusters (AuNPs/NCs); (b) quantum dots (QDs)[15–20]; (c) polymer-based nanomaterials (conductive polymers, polymer dots etc.)[21–25]; and (d) rare earth metal-based nanoparticles (upcon-version nanoparticles).[26,27]

AuNPs have been widely used as imaging agents in cancer diagnosis and tumor detection. AuNPs display the surface plasmon effect. [13,28–30] Interaction between light and AuNPs creates dipoles on the Au surface, and subsequently absorption peaks in the visible wavelength range.[31] The high surface electron density characteristic of AuNPs, makes them suitable as powerful quenchers in fluorescence imaging. AuNPs can be used as “on–off;” fluorescence probes in imaging of intracellular biomolecules.[32] Although gold nanoclusters (AuNCs) with particle size of less than 2 nm do not have suitable absorption in the visible wavelengths, they have good stability for imaging and detection of cancerous cells and different intracellular biomolecules.[33] AuNPs are highly efficient quenchers that can be applied in different nanoflare-based intracellular imaging approaches. The efficient quenching ability of AuNPs makes it possible to use different dyes as molecular beacons in intracellular environments.[34] Modified AuNPs can detect a broad range of nucleic acids,[35] adenosine triphosphate (ATP),[36] cancerous cells[37] and several intracellular ions.[38] Moreover, different dye molecules can be used as beacons in nanoflare-based intracellular imaging. The optical properties of various organic dyes that have been applied as beacons in intracellular biomolecular detection have recently been reviewed.[39] The emission wavelength of fluorescent dyes is a critical factor that should overlap with the surface

plasmon resonance of modified AuNPs. The matching of the absorption wavelength of the AuNPs and the emission wavelength of the dye plays a very important role in the “on–off” fluorescence properties of nanoflares.[40,41] Currently, many scientific reports have described intracellular detection of a variety of targets using nanoflares, detecting a broad range of RNAs, cations, cancerous cells etc. The great potential of nanoflares can improve the imaging and detection of cancerous cells, and assist in elucidating their unpredictable intercellular pathways. Applications of nanoflares to the detection of RNA, DNA, cancerous cells, intracellular components, and biomolecules will be discussed in the subsequent sections.

Hybrids between AuNPs (13 nm) and oligonucleotides are the basic building block of nanoflares. The dense deposition of oligonucleotides on the AuNP surface makes them suitable for fluorescence-based detection. The fluorophore emission wavelength, the configuration and morphology of the oligonucleotides on the AuNP surface, and the distance dependent plasmonic resonance are all critical features.[42–45] Many different types of fluorophore have been tested including, Cy5, Cy3, Texas red, and rhodamine 6G. These dyes are stable against enzymatic degradation, and their emission wavelength can overlap with AuNP surface plasmon resonance absorption peaks.[5] The “off–on” generation of fluorescence from nanoprobes has been developed. The specific structural design of the oligonucleotides makes it possible to release fluorophore molecules during the hybridization process between targets and probes. The size of AuNPs bears a direct relation with their biological toxicity. Smaller AuNPs are generally more toxic than large AuNPs. The toxicity was found to be negligible for AuNPs 50 nm in size, but AuNPs with 13 nm size are commonly used in this application.[46,47] Moreover, increasing the size of AuNPs increases the specific surface area and hence the number of oligonucleotide molecules that can be immobilized.

From a clinical point of view, the ability of nanoflares be employed in vivo is one of the important issues that will determine their eventual acceptance, There are many factors that are important for in vitro experiments, but more importantly it is necessary to study nanoflares under in vivo conditions. The cytocompatibility, long blood circulation time, specificity to the target and low toxicity to the surrounding tissue are the most important features in designing nanoflares. According to recent reports, the size of metal nanoparticles and the length of the decorated oligonucleotides are the most important structural features. Commonly, metal nanoparticles with larger sizes (up to 50 nm in diameter) and oligonucleotides with shorter length (25–90 bases) show the best results for in vitro conditions. But larger metal nanoparticles have a low specific surface area, and subsequently show lower contrast in imaging applications. Therefore, researchers have proposed that nanoparticles with smaller sizes (13 nm diameter) will improve the contrast in vivo. Moreover, metal nanoparticles with 13 nm size have acceptable performance under in vitro conditions. Unlike short oligonucleotides (25–90 bases) that are suitable for in vitro conditions, longer oligonucleotides are more appropriate for in vivo condition. This issue affects the sensitivity, limit of detection, and background signals from the probes in the intracellular milieu.[48,49] Appropriate structural design, chemical stability, suitable size, and low cytotoxicity are desirable properties. So, in summary, the concept of “nanoflares” is relatively new, but is becoming increasingly widely adopted. Nanoflares have some

structural features that are crucial to produce photoluminescence used for intracellular detection of a range of important analytes. A nanoflare is a nanocarrier with photoluminescence detection ability that has been constructed from nanoparticles with a plasmon resonance frequency on their surface, and decorated with oligonucleotides as biological recognition elements. In fact, the nanoflares have dual features that make them suitable options for detecting or imaging a range of intracellular physiochemical events, or intracellular analytes. Nanoflares have special optical properties that depend on the plasmon resonance frequency of the nanomaterials such as AuNPs, AgNPs etc., coupled with appropriate dyes or beacons with an emission wavelength that should be matched with the plasmon resonance frequency of the metal nanoparticles. Some useful properties of the nanoflares that make them more attractive than conventional fluorophores for intracellular detection are the nanometer scale of their structure that facilitates their transfection process through the cellular membrane and into the cells; high chemical stability in a harsh enzymatic intracellular milieu coupled with low toxicity and high biocompatibility. According to these explanations, the nanoflares can be selected as promising option for detection of range of range of mutated genes or biomarkers related to serious diseases such as cancer, Alzheimer's etc. In the next sections, the molecular targets for nanoflare-based imaging and detection inside cells are discussed.

Targets in nanoflare-based imaging

Detection of cancer cells

We know that quenching process and the emission wavelengths of the dyes or beacons are important for intracellular detection of analytes. Nanoflares provide a chance to achieve bioimaging arising from intracellular events or processes. Some diseases have an underlying genetic cause, and the detection of defective cells, and their location within the human body is a complicated and difficult process. Nevertheless, photoluminescence-based methods can reveal altered cells with great precision and accuracy and high specificity. Cancer is one of the most dangerous diseases caused by mutated and defective genes, allowing cells to undergo unbridled divisions, followed by spreading and metastasis throughout the human body. The detection of cancer biomarkers using nanoflare-based methods is a highly potent strategy that may be suitable for commercialization because of high accuracy, low detection time, and an efficient transfection through cell membranes into cells. So, suspicious pathological samples can be tested using lab-on-chip type devices in clinical applications. Moreover, from a technical and spectroscopy point of view, we have been limited by the Hb and HbO_2 absorption wavelength which lies between 500 and 600 nm and can affect the contrast in optical bioimaging. Therefore, the ability of nanoflares to match the emission wavelength of the beacons with the plasmon resonance frequency of the nanoparticles is a rare and unique feature. Despite decades of effort, early detection and effective cancer therapy remains a major challenge. Many different biological molecules have been explored as biomarkers of cancer. According to many reports, these biomarkers mostly consist of genes,[50–55] ribonucleic acids (RNAs),[56] carbohydrates[57–59] and glycoproteins.[60–66] Many of these biomarkers are present at only low concentrations and their detection is complicated. Moreover, early diagnosis of cancer often requires detection of these biomarkers at low concentrations inside cells, rather than in biological fluids. Nanomaterial-based methods

have high sensitivity, accuracy and low limits of detection (LOD) for cancer biomarker detection. QDs and modified QD-based nanomaterials[67] fulfill many of the requirements for cancer cell imaging.[68] QD-based methods can be used in “lab-on-chip” approaches.[69,70] Fluorophore-labeled nanomaterials have been employed for detection of biomarkers related to gastric cancer.[71,72] AuNPs have good quenching properties and low levels ofbackground fluorescence. Physical interactions between AuNPs and phosphatase isoenzyme biomarkers can cause the release of absorbed organic quenched beacons to detect the presence of cancer cells (Figure 1).[73] (See Table 1.)

Detection of cancer cells can involve cancer metastasis or circulating cancer cells,[74–76] proteins expressed on the cell surface,[77–80] or gene expression-based methodology.[81–83] Nanoflares are an important and highly efficient method of detecting changes in gene expression. Detection of different RNAs has been achieved using AuNPs with attached oligonucleotides. Prigodich et al. developed hybrid-based nanoflares consisting of AuNPs with fluorophore-labeled oligonucleotide (DNA-LNA chimeras) as theranostic agents for detection of HeLa (human cervical cancer) and C166 (mouse endothelial) cells. The marker that was targeted was messenger ribonucleic acid (mRNA) encoding for endogenous survivin, an anti-apoptotic protein over-expressed in many cancers. A schematic illustration of the use of AuNP-oligonucleotide nanoflares for detection of survivin mRNA in HeLa and C166 cancer cells is shown in Figure 2. The hybridization process between survivin mRNA and nanoflares resulted in an increase in the intensity of Cy5 dye fluorescence.[84]

The detection of abnormal RNAs within cells located in different parts of the human body is a challenge. The importance of mRNA expression levels for cancer detection has led researchers to concentrate on multiple RNA detection methods.[37] Cellular metabolism and progression,[85,86] activation,[87–90] and ganglioside pathways[18,91] are all controlled by different mRNAs, and disruption in their levels can be related to the presence of cancer. Li et al. devised a detection method for multiple tumor-related mRNAs based on nanoflares with multicolor optical emissions. They used AuNP-oligonucleotide hybrids as nanoflares for simultaneous detection of mRNAs coding for TK1 (human thymidine kinase), c-myc (oncogene) and GalNAc-T (N-acetylgalactosaminyltransferase) in breast cancer cells. TK1, c-myc and GalNAc-T all play key roles in tumor cell progression. The nanoprobes used the following fuorochromes: green (Rh110), yellow (Cy3) and red (Cy5) colors. The response of the combined nanoflares was up to five times higher than the response of single ones. The background fluorescence signals did not change in the presence of irrelevant intracellular biomolecules. The LOD of these nanoprobes was 1.6 nM, 1.4 nM and 1.2 nM for GalNAc-T, TK1 and c-myc mRNAs, respectively. The nanoprobes had high stability against nuclease, deoxyribonuclease I, and endonuclease enzymes (Figure 3).[83]

During the metastatic process, cancer cells can be distributed all over the body before the appearance of discrete lesions. The transition from the epithelial to mesenchymal cell phenotype (EMT) governs the spreading of cancer cells to distant organs. Typically, reduced expression of adhesion molecules (such as, E-cadherin, fibronectin and vimentin) leads to loss of intercellular contacts[92] leading to cell migration to other parts of the body.[93] Halo et al. devised a nanoflare-based approach to detect mRNAs related to EMT in metastatic cancer cells. The mRNAs coded for the mesenchymal markers, twist, vimentin, and

fibronectin, and the epithelial marker E-cadherin. The nanoflares consisted of a spherical AuNP densely functionalized with a monolayer of ssDNA attached via a 3′ thiol group that was complementary to mRNAs for the target genes. The ssDNA “recognition sequence” was prehybridized to a shorter DNA complement containing a fluorescent reporter (the “reporter flare”) whose fluorescence was quenched based on its proximity to the AuNP. When target mRNA binds the recognition sequence, the reporter flare strand was displaced, liberating the fluorescent Cy5 dye.[94]

Prigodich et al. reported multiplex nanoflares that could detect HeLa, Jurkat, and MCF-7 human cancer cells using commercial siRNAs (small interfering RNAs). Their results indicated that the nanoflares showed a high ability to detect HeLa, Jurkat, and MCF-7 cell mRNAs using actin and survivin as complementary sequences. They used Dharma-FECT 1 transfection reagent in the imaging process. The multiplexed nanoflares and their different detector sequences showed independent behavior in buffer media. A schematic representation of simultaneous and independent intracellular imaging of mRNAs in human cancer cells using multiplex nanoflares is shown in Figure 4.[95]

Pan and coworkers developed AuNP-based nanoflares with four color probes for simultaneous detection of mRNAs. The intracellular detection of TK1, survivin, c-myc and GalNAc-T related mRNAs were possible. They used HepG2, MCF-7, MCF-10A and HL-7702 cancer cells. A schematic of the four color-based nanoflares is shown in Figure 5.[96]

It is important to minimize the thermodynamic fluctuation in order to remove unwanted false positive signals. Yang et al. fabricated AuNP-based nanoflares with acceptor-donor pairs that could detect mRNAs in HepG2, MCF-7 and L02 cancer cells using a fluorescence resonance energy transfer (FRET) system (Figure 6). They utilized the TK1 mRNA as the main target in cells. They found that intracellular DNAse I had an important influence on the optical properties of nanoflares by cleaving the nucleotide sequences of both target and flare. DNAse I had a remarkable effect on the fluorescence background optical signal.[97]

An abnormal level of telomerase enzyme activity is another marker of cancer that can distinguish malignant cells from normal ones. The telomerase enzyme is responsible for adding telomere units onto the end of chromosomes. Telomerase controls proliferation and cell division and effectively makes cells “immortal”.[98] Hong et al. described AuNP-graphene oxide (GO) hybrid-based nanoflares for in situ detection of telomerase activity in cancer and normal cells. They used this novel nanoflare-based method to analyze HeLa, A549, MCF-7, Caco-2, HBL-100 and HL-7702 Cells. The high transfection efficiency, enzyme-free detection, and the amplification process were claimed as the main advantages of this novel nanoflare-based method. A schematic illustration of AuNPs-GO hybrid-based nanoflares is shown in Figure 7.[99]

In another study, He and co-workers, developed a ratiometric fluorescent-based biosensor for detection of cancer cells based on differences in telomerase expression levels. They prepared modified AuNP-based nanoflares with oligonucleotides to detect telomerase mRNA in cancer (HeLa, HepG2, A549, and 293 T) and normal (QSG) cells. Their results

indicated that the ratiometric nanoflare probes could detect telomerase levels via the complementary mRNA sequences. A schematic illustration is shown in Figure 8.[100]

MicroRNAs (miRNAs) are biomarkers that have been used as for cancer cell detection. miRNAs are related to the malignant properties ofcells such as a disruption in the transcription process, uncontrolled epigenetic processes, and defects in protein synthesis.[101] Hong et al. prepared nanoflares using a hybrid between AuNPs and GO with oliogonucleotides for detection of mature/pre-miRNA-21 inside cells. Their results suggested that nanoprobes had an excellent LOD and a linear range of activity. Expression levels of pre-miRNAs were unchanged, but levels of mature miRNAs expression showed a significant change in cancer cells. Whether the expression levels were increased or decreased depended on the nature of the miRNA. Mature miRNA-21 was upregulated and mature let-7a was down-regulated.[102]

Nanoflares based on Au-Se bonds have also been used in various biosensing systems. They are particularly useful in intracellular environments, in which high concentrations of free thiols (such as reduced glutathione) would destroy Au-S bonds, and lead to high background signals. Gao et al. developed surface-modified AuNPs using selenol peptides as linking agents, for the detection of the matrix metalloproteinase 2 (MMP-2) enzyme with remarkable stability and low chemical anti-interference. Comparison between Au-S and Au-Se nanoflares indicated that the Au-Se bonded one performed better than the nanoflare with Au-S bonds due to higher stability in the presence of glutathione.[103]

Cancer therapy

Nanoflares can have applications in cancer therapy as well as in cancer detection. Combining drug delivery methods with nanoflare-based systems can provide novel clinical approaches to cancer treatment. Kyriazi and co-workers developed a multiplex nanoflare-based drug delivery system using hybrids between DNA and AuNPs prepared by a self-assembly method. Two anticancer drugs were tested, mitoxantrone (MTX) and doxorubicin (DOX). Two separate AuNPS were linked together via DNA linkers, with attached sense strands of ssDNA that recognized mRNAs in living cells, and flare strands that were released upon binding to the target. The high surface area of oligonucleotide-modified AuNPs made it possible to load large amounts of drug, and specifically release it upon binding to the target, with the nanoflare as a fluorescent reporter. This multiplex nanoflare system is shown in Figure 9.[121]

The human mutT homologue (MTH1) is an oxidized purine nucleoside triphosphatase enzyme, which has been explored as a cancer marker. Simultaneous detection and inhibition of MTH1 activity could be beneficial in cancer therapy. Gao et al. developed AuNPs bound to mesoporous silica nanoparticles (MSNPs)-as nanoflares for detection and inhibition of MTH1 inside living cells. They used S-crizotinib (an aminopyridine drug) as a MTH1 inhibitor that was loaded onto the capped hybrid MSNP-AuNP nanoflare-based system. Release of S-crizotinib inside live cells was triggered by the hybridization process with the MTH1 mRNA. The rate of S-crizotinib release depended on the MSNPs pores that opened when the nanoflares hybridized to their target. They tested this novel method for different cancer types such as kidney, liver, and lung using both in vitro cultured cells (HeLa, HepG2

and HL7702 cancer cells), and in vivo in xenografted mouse tumors. Significant reductions in tumor size were observed. These MSNPs-AuNPs-based nanoflares are shown in Figure 10.[122]

The shapes and sizes of different nanomaterials,[123,124] and their interfacial properties[125] have large effects on their interactions with different tissues and organs. AuNP-DNA hybrids can take advantage of nucleic interactions to tailor the shape and size of the nanostructures, and have been termed “satellite-like”.[126,127] Raeesi et al. reported satellite-like nanoflares using AuNPs modified with DNAs for efficient drug release. Intelligent design of these satellitelike nanoflares made it possible to manage the interactions between drug molecules and their carrier. The preparation and design of different parts of the satellite-like AuNP-based nanoflares is illustrated in Figure 11.[128]

Adenosine triphosphate (ATP) detection

ATP is an important biomolecule with a key role in energy metabolism, intracellular biological pathways, and cellular activity. The natural range of ATP concentrations inside cells varies from 0.1–3.0 mM. Zheng et al. modified AuNPs with an RNA aptamer sequence to specifically detect ATP inside lysosomes.[129] Jin et al. developed a nanoflare-based sensor with subcellular detection capability. According to the different ATP concentrations inside cells (<0.4 mM), and in the extracellular environment (1–10 mM),[130] their AuNP-modified nanoflares had the appropriate sensitivity and activity for detection. The presence of other ATP-like biomolecules such as: uridine triphosphate, guanidine triphosphate and thymidine triphosphate did not interfere with the nanoflare-based sensor.[131]

pH Sensing

The pH levels inside cells can control different biological pathways. pH levels have been implicated in drug resistance, transportation of different ions, regulation of biological systems, membrane dynamics, and cell division and proliferation.[132–135] Moreover correlations have been established between intracellular pH and myocardial ischemia,[136] Alzheimer’s disease[137] and many forms of cancer.[138] “i-motif” biosensors have been constructed from ssDNA that have an intrinsic ability for detection of intracellular pH.[139] At high pH, the fluorescent dye is hybridized and quenched, but at low pH the fluorescent probe sequence is released. Huang et al. modified ssDNA-AuNP-based nanoflares that could sense pH inside living cells. Changing the structural conformation of the i-motifs, made it possible to measure pH values ranging from 5.0–7.0. The pH-independent properties of the fluorophores, ensured that pH changes from 7.5–5.0 had no effect on the fluorescence intensity. Figure 12 shows a schematic illustrations of the i-motif for pH measurement.[140]

Inorganic ion detection

The intracellular levels of inorganic ions can affect numerous disease conditions.[141] Zn^{2+} ions are involved in gene transcription and the transmission of neural signals.[142,143] Cu^{2+} ions can regulate and control the mitochondrial respiratory function within cells, by modifying enzyme activities, and can modulate the immune system.[144,145] Excessively high levels of Cu^{2+} can produce harmful effects such as disruption of neural systems, kidney and liver failure,[146,147] and even contribute to prion-based diseases.[148] Simultaneous detection

of Zn^{2+} and Cu^{2+} inside cells could therefore be attractive. Nanoflare-based biosensors can be a powerful tool for the early-detection of biomarkers of Alzheimer's disease and prion-based diseases in the human body. These biomarkers are often related to unbalanced levels of metal ions like Zn^{2+} and Cu^{2+}. Li and co-workers designed a AuNP-DNAzyme nanoflare-based method for measurement of Zn^{2+} and Cu^{2+} ions in living cells. DNAzymes are specific for Zn^{2+} and Cu^{2+} ions, comprising substrate strands labeled with fluorophores at the 5′ end and quenchers at the 3′ end. The fluorescence of the fluorophores is quenched both by the gold nanoparticle and the quencher. After the nanoprobes are transferred into the cells, the substrate strands would be cleaved in the presence of the Zn^{2+} and Cu^{2+} target ions, resulting in disassociation of the shorter DNA fragments containing fluorophores. These multiplex nanoflares had high enzymatic stability and the ability to monitor and image Zn^{2+} and Cu^{2+} ions. They showed a response at Zn^{2+} and Cu^{2+} concentrations from 0 to 5.0 μM. The linear ranges were for Zn^{2+} (1.0–30.0 nM) and for Cu^{2+} (1.0–20.0 nM). The LOD was 0.47 nM and 0.45 nM for Zn^{2+} and Cu^{2+} ions, respectively. Other ions (Mn^{2+}, Cd^{2+}, Mg^{2+}, Ni^{2+}, K^{2+}) did not show undue interferences because the nanoflares showed high optical signals for their target Zn^{2+} and Cu^{2+} ions. The use of AuNP-DNAzyme nanoflares for Zn^{2+} and Cu^{2+} ion detection is shown in Figure 13.[149]

K^+ ions play a unique role biological in the metabolism of the heart and in hyperpolarization of cells in the nervous system.[150,151] Yang et al. developed a FRET-based nanoflare system composed of AuNPs modified with G-quadruplex oligonucleotides for intracellular K^+ ion detection. The K^+ ions can interact with the G-quadruplex oligonucleotides which are dual-fluorophore-labeled so that FAM acts as a donor and TAMRA as an acceptor. The complementary oligonucleotides are designed to bind with the G-quadruplex sequences (flares) and immobilized on the AuNP surface via an Au–S bond. In the absence of target K^+ ions, the G-quadruplex sequences bind to the complementary strands, separating the donor (FAM) and acceptor (TAMRA), and destroying the FRET coupling. In this open state, only the fluorescence of the donor dyes can be detected. However, in the presence of target K^+, the flares are gradually displaced from the complementary strands, subsequently forming G-quadruplex structures that bring the donor and acceptor into close proximity and result in a high FRET coupling efficiency. In this closed state, the fluorescence of the acceptor can be detected when the donor is excited. Thus, the fluorescence emission ratio of acceptor to donor (A/D) can be used as a signal for measurement of target K^+. Sodium ions (Na^+) could interfere with assays for K^+ ions, but for the modified AuNP-aptamer nanoflares the sensitivity for K^+ ions was 4.5-fold higher than other ions. A schematic description of the AuNP-aptamer nanoflare system is shown in Figure 14.[152]

Outlook and future perspectives

Nanoflares have become an exciting new alternative to more traditional intracellular fluorescence probes. They are able to enter cells relatively easily and detect the target genes (or other analytes) with high sensitivity and selectivity. Future work should be undertaken to explore the wider possibilities of using nanoflares for the detection and analysis of many genetic-based diseases in addition to cancer. Furthermore, we anticipate that nanoflares will have applications for investigating RNA functions inside cells, and its dysregulation in diseases. Nevertheless, knowledge about the intracellular trafficking of nanoflares at the

molecular and tissue levels is still relatively sparse. More studies on the subcellular localization, transport from cell to cell within living systems, and penetration into tissues and organs are required.

Conclusion

Optical nanoflare-based imaging systems are becoming an efficient detection approach for many intracellular biomolecules. AuNPs modified with oligonucleotides can function as nanoflares and fluorescent biosensors with a high transfection capability, good enzymatic stability, appropriate surface plasmon resonance absorption peaks, and ability to match the emission wavelength. Their high selectivity and specificity could overcome weaknesses of traditional detection methods. The main topic of this review focused on the range of different targets that the nanoflares could detect inside cells. The ability to detect intracellular biomolecules is important for early-diagnosis of a range of diseases especially cancers, and for increased understanding of cancer cell signaling pathways. Nanoflares can be used to monitor intracellular drug delivery methods, and even to increase the efficiency of drug delivery. The structural and optical features of nanoflares are just starting to be fully understood, and their properties must be optimized for a diverse range of applications. These applications have so far have included the detection of a wide range of mRNAs (many of which are related to cancer such as EMT and telomerase), DNA expression, ATP levels, and levels of various inorganic ions inside cells. The unique design of nanoflares makes it possible to detect ultra-low concentrations of biomarkers in the complicated intracellular milieu. Interference between the target and other similar biomolecules was an unsolved challenge for many years, while nanoflares have helped to solve this problem. We predict that nanoflare-based fluorescence methods will continue to play a remarkable role in medical and biological sciences in the future.

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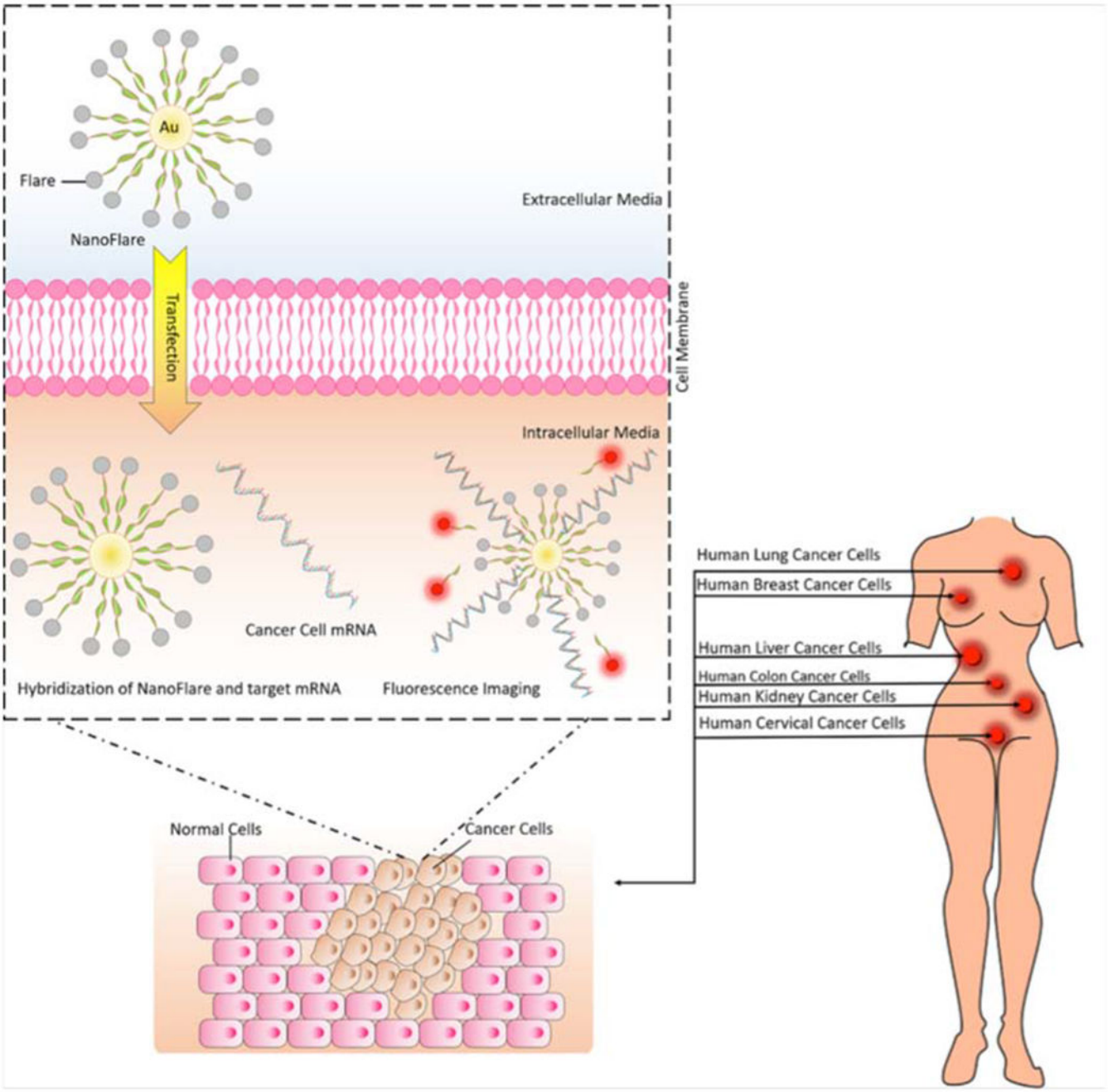

Figure 1.
Application of nanoflares for detection of abnormal mRNA in cancer cells.

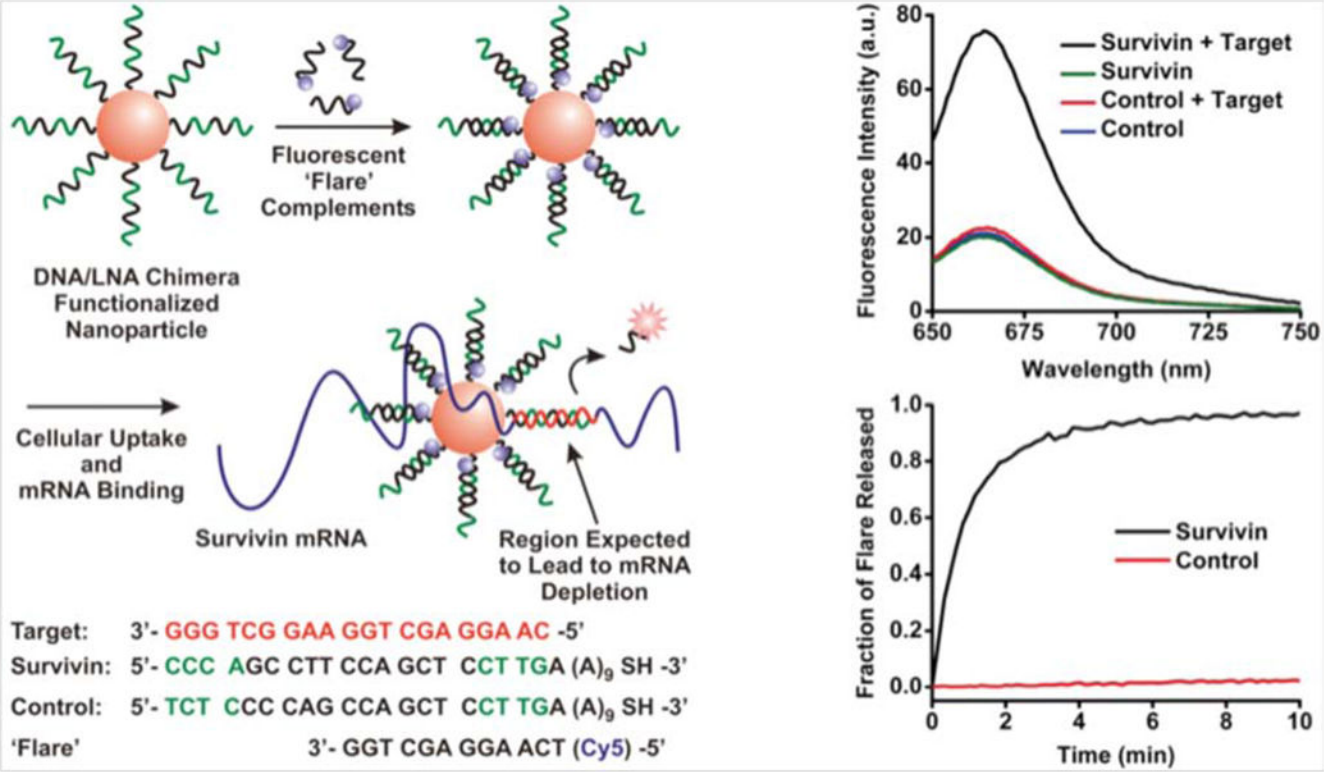

Figure 2.

Surface-modified AuNP nanoflares and their fluorescence intensity change (upper plot). Transfection of the nanoflare inside cells, interaction between survivin mRNA and the nanoflare, and released flare was monitored over time (lower plot)[84] with permission.

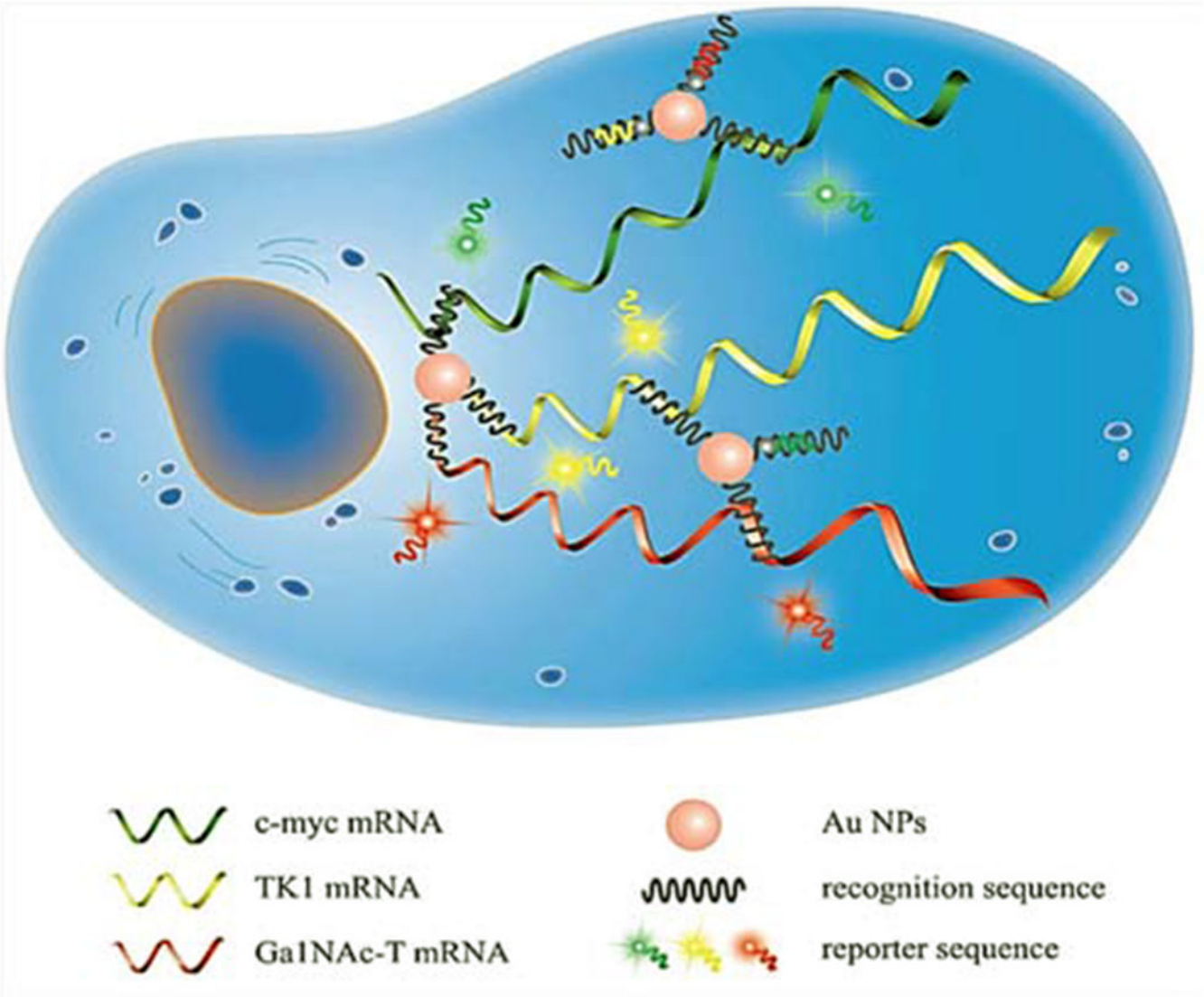

Figure 3.

Illustration of simultaneous detection of c-myc, TK1 and Ga1NAc mRNAs in human breast cancer cells (MCF-7, MCF-10A) and HepG2, HL-7702 cells using AuNP-oligonucleotide nanoflares[83] with permission.

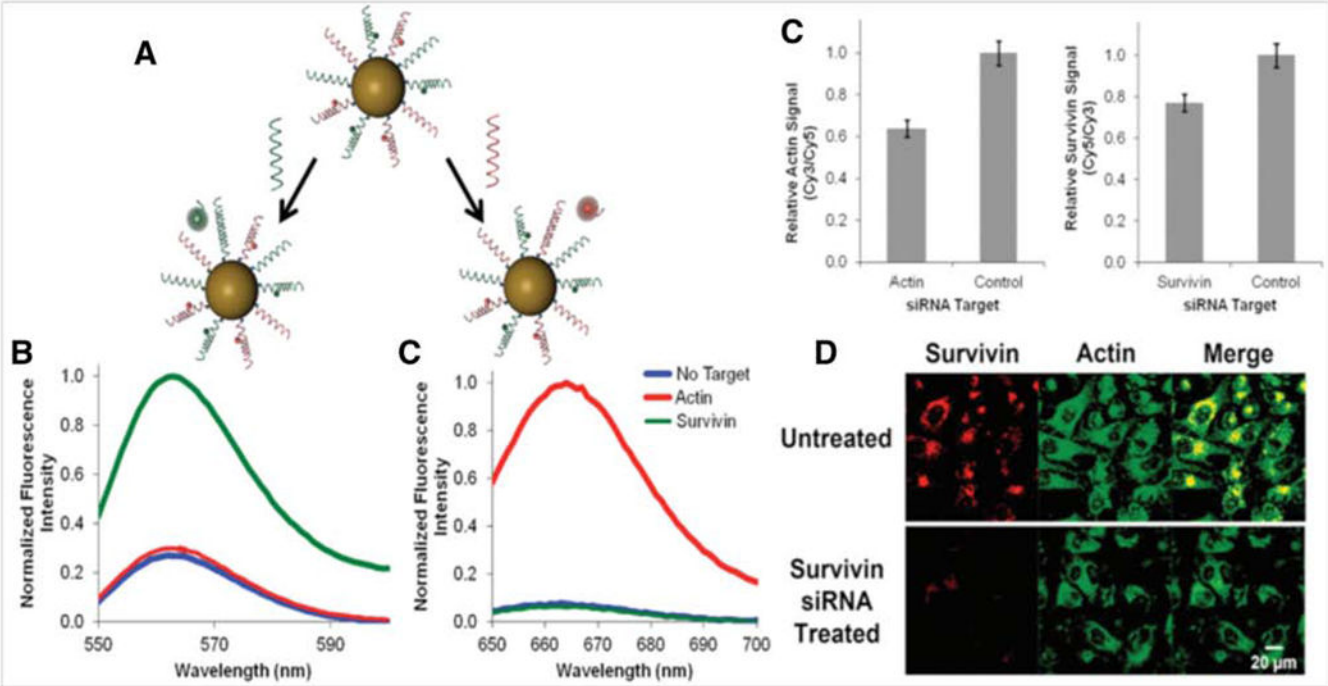

Figure 4.

(A) Spherical AuNP-based nanoflares and detection process of different cancer cell mRNAs, (B) Fluorescence spectrum of actin and survivin based nanoflares with independent fluorescence wavelengths, (C) Actin and survivin relative fluorescence signals using Cy3 and Cy5 dyes, and (D) fluorescence confocal microscopy (FCM) images of survivin and actin mRNAs, and the combination, for intracellular imaging in human cancer cells[95] with permission.

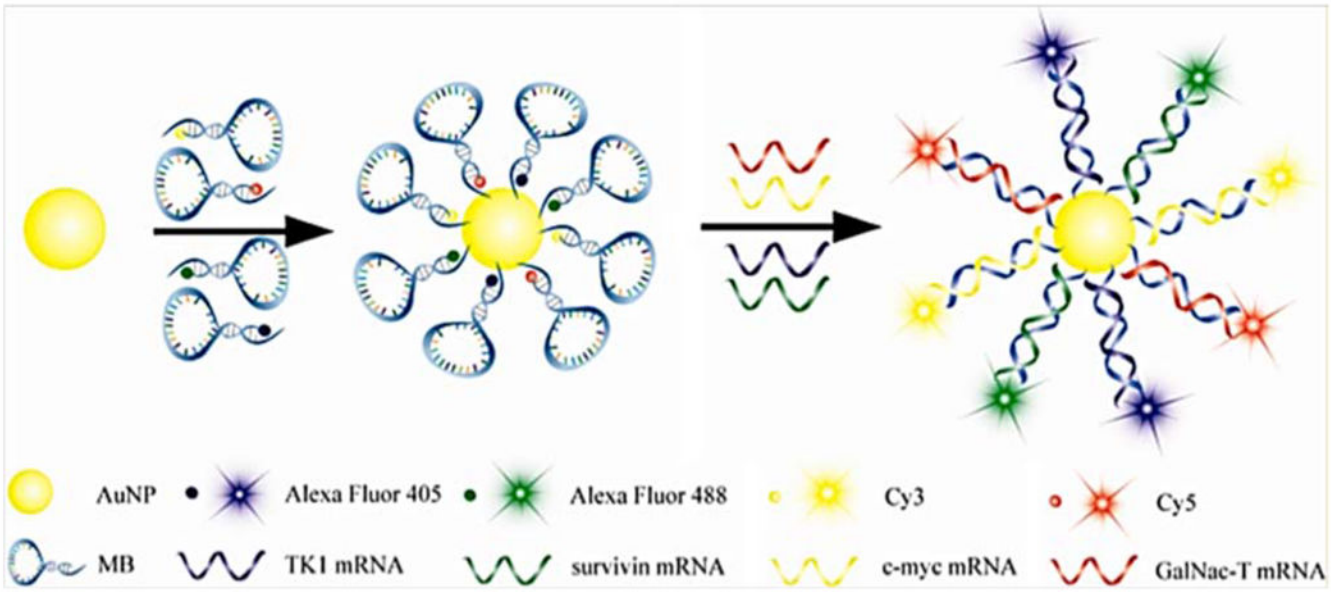

Figure 5.

Molecular mechanism of multiplex four color AuNP-based nanoflares for detection of TK1, survivin, c-myc and GalNAc-T mRNAs in cancer cells[96] with permission.

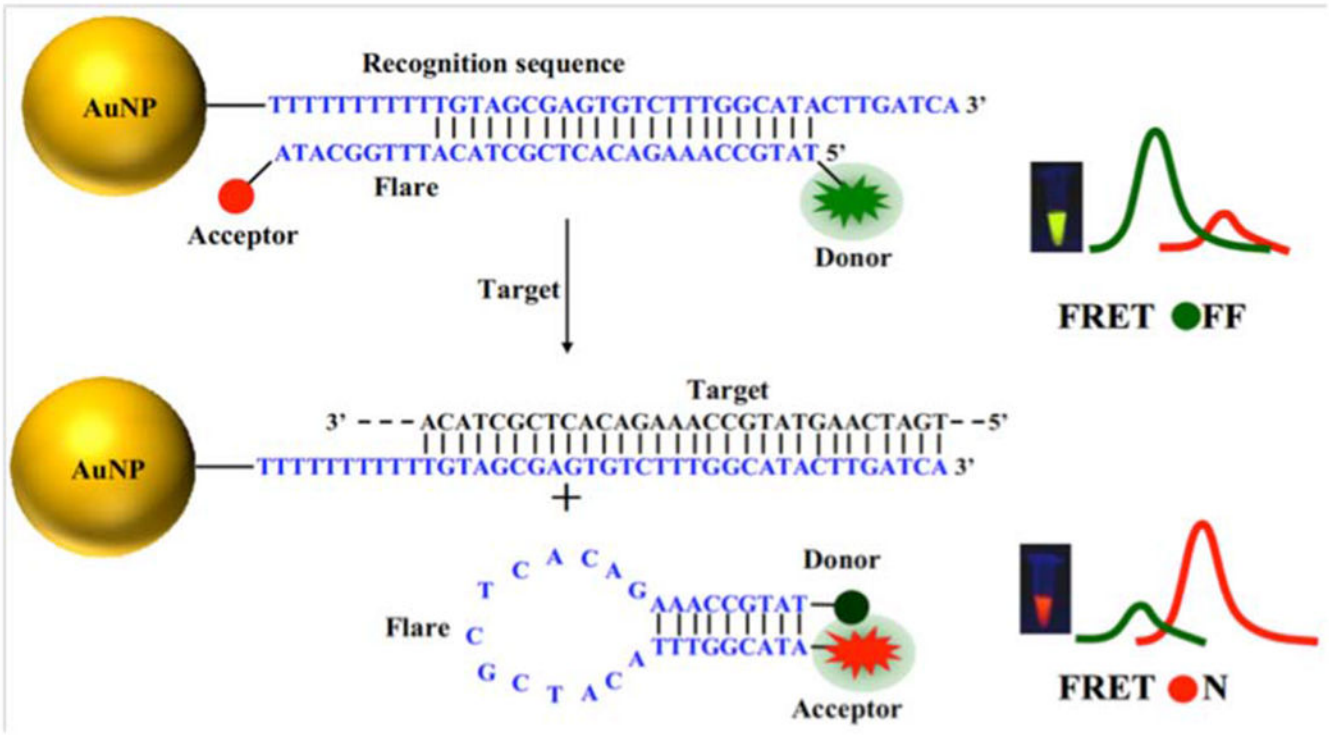

Figure 6.
AuNPs-based nanoflares with acceptor-donor flare beacon[97] with permission.

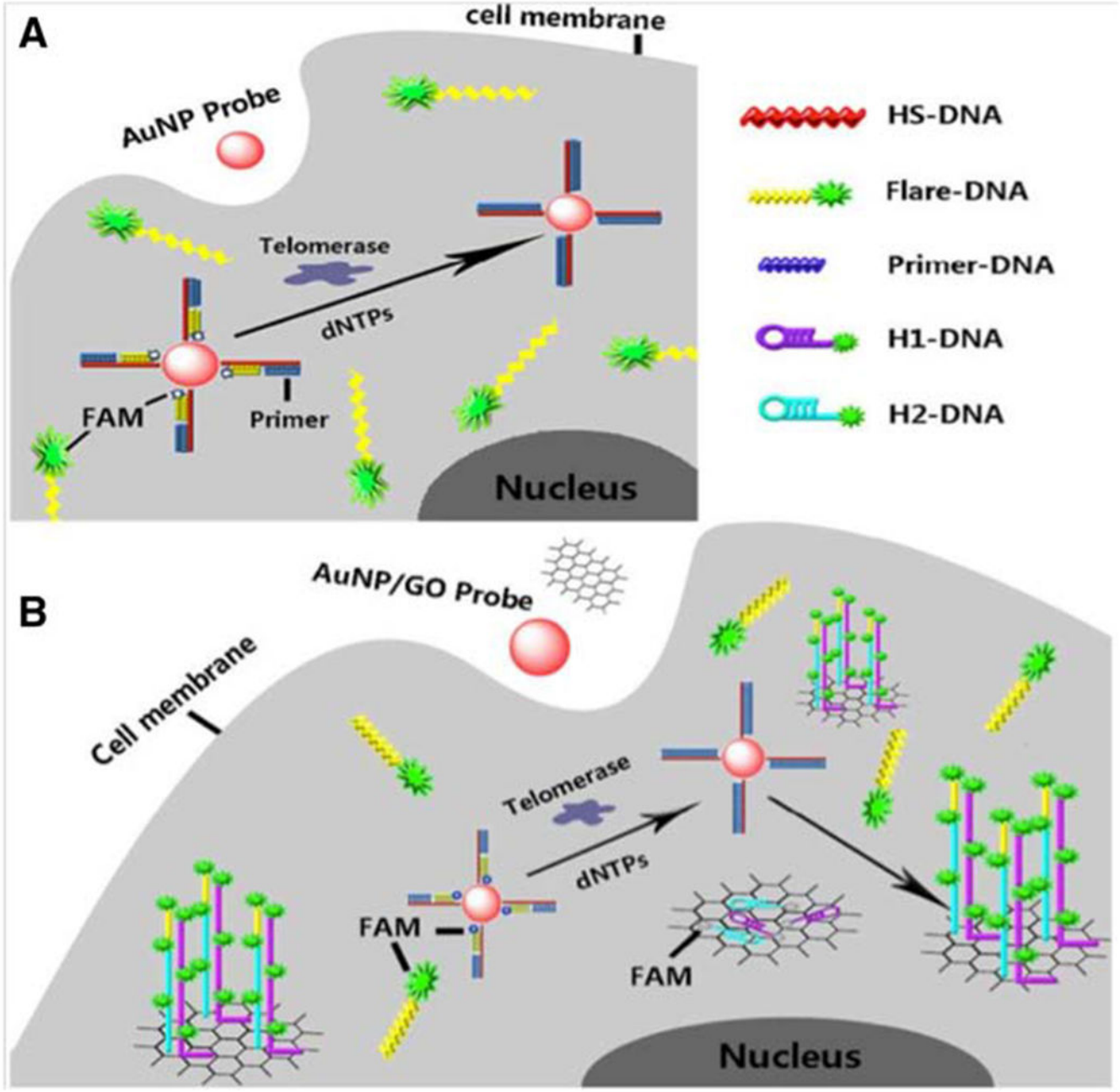

Figure 7.
Schematic representation of AuNP-GO hybrid-based nanoflares[99] with permission.

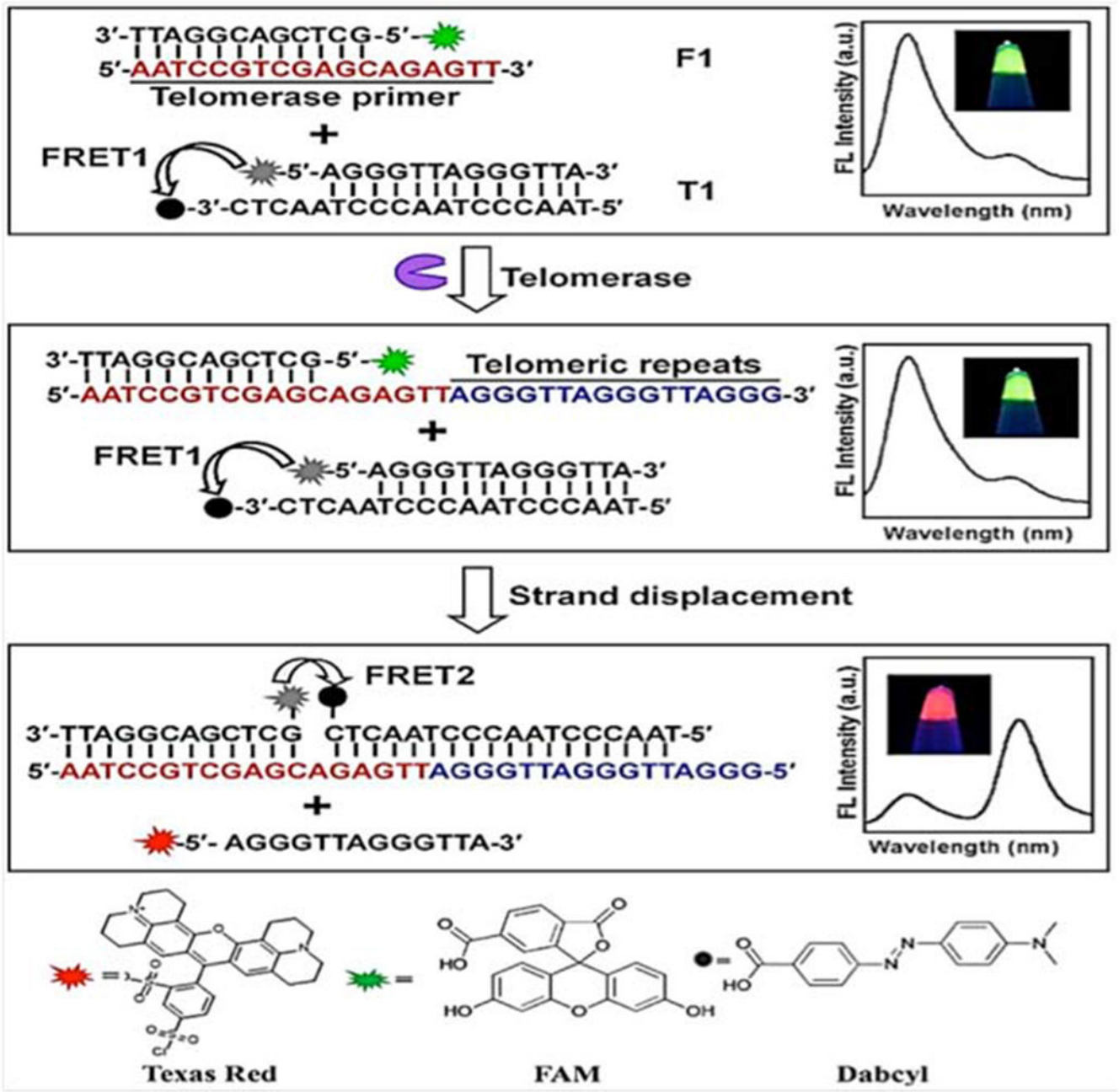

Figure 8.

Telomerase activity detection using ratiometric nanoflares[100] with permission.

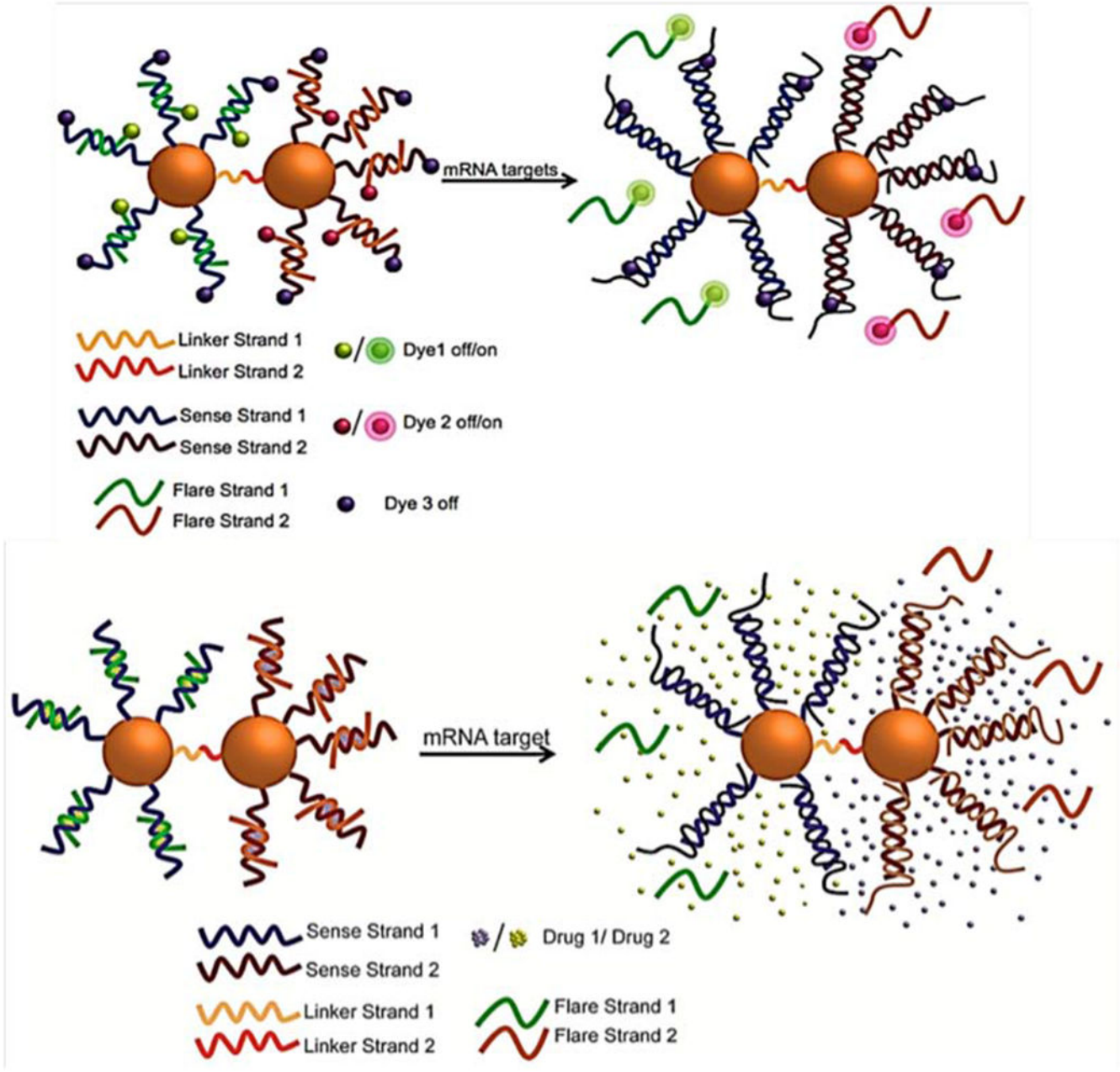

Figure 9.

Multiplex dimeric AuNPs and DNAs hybrid-based nanoflares with fluorescent dyes Cy3, Cy5 and FAM have been used in flares. Simultaneous drug loading (MTX and DOX) on the nanoflare and delivery inside cells for anticancer therapy[121] with permission.

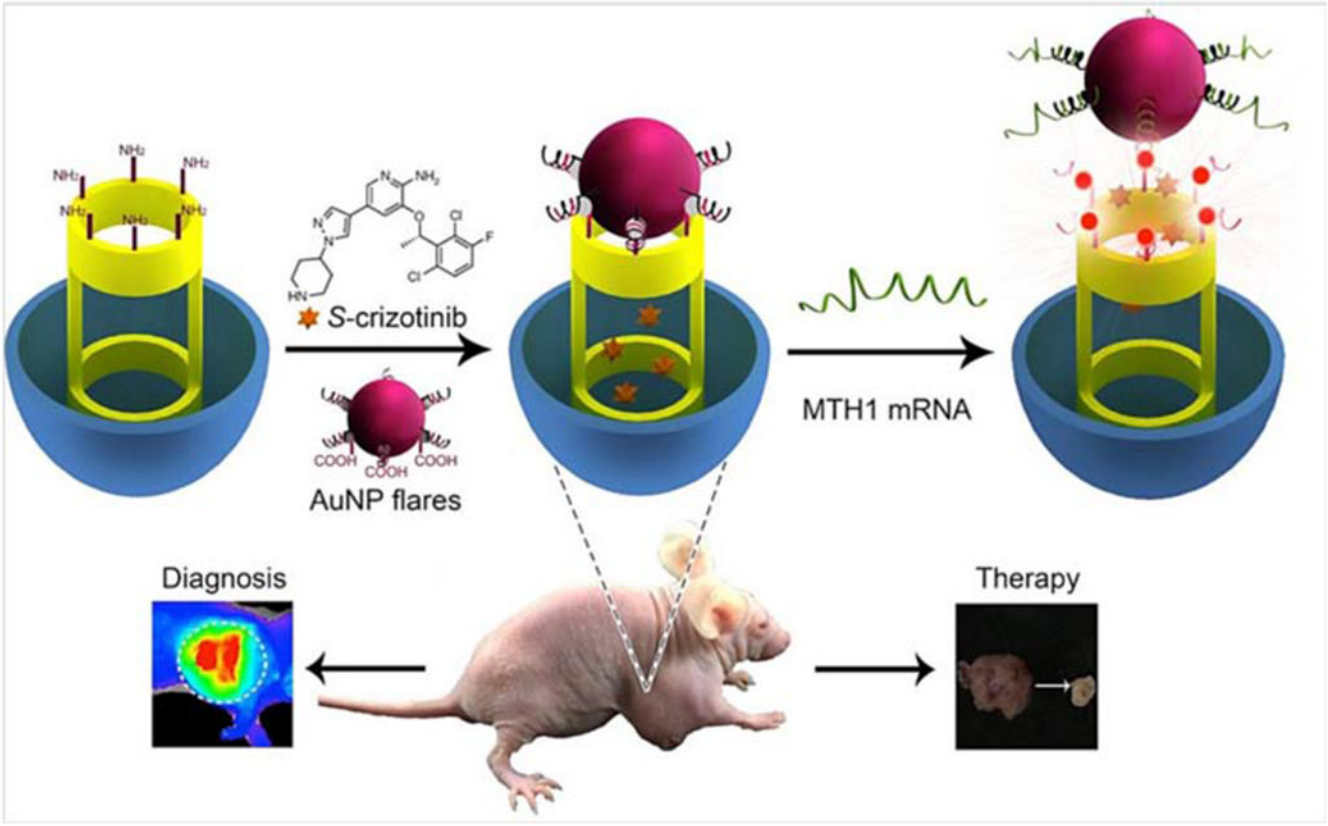

Figure 10.
Schematic illustration of capped MSNP-AuNP nanoflares for MTH1 detection and inhibition in cancer therapy[122] with permission.

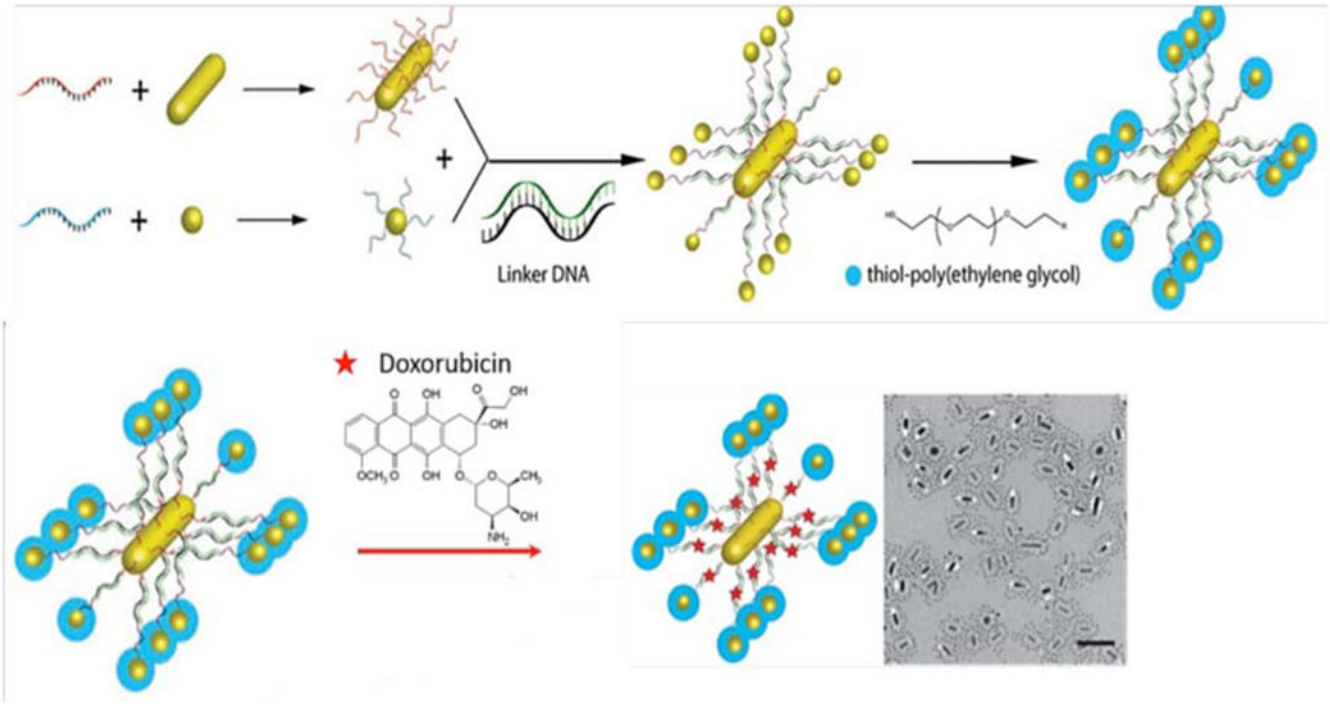

Figure 11.

Gold nanorods (AuNRs) and AuNPs have been used in satellite-like nanoflares designed for loading with doxorubicin anticancer drug (upper panel), transmission electron microscopy (lower panel)[128] with permission.

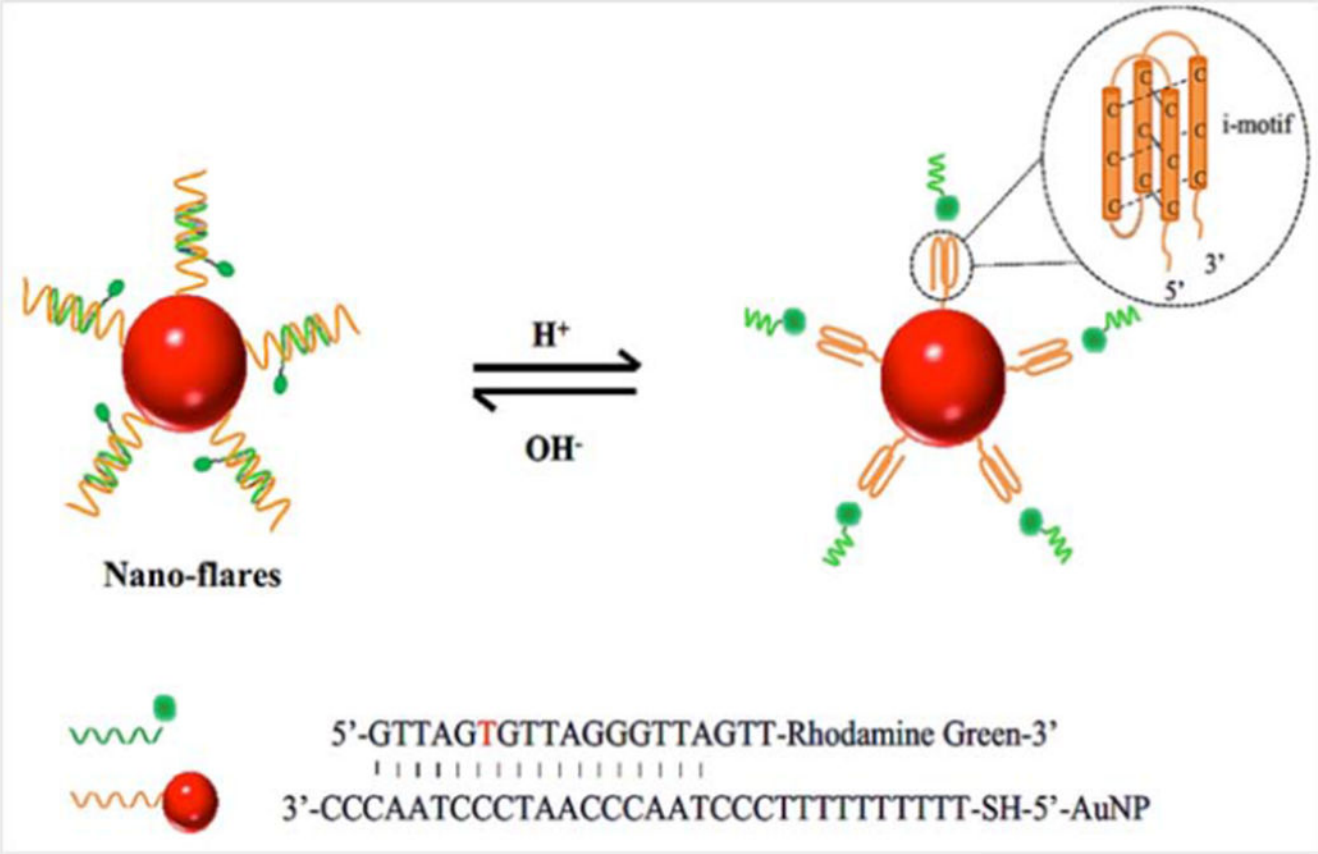

Figure 12.
Schematic illustration of pH dependent i-motif-based nanoflares as pH biosensors[140] with permission.

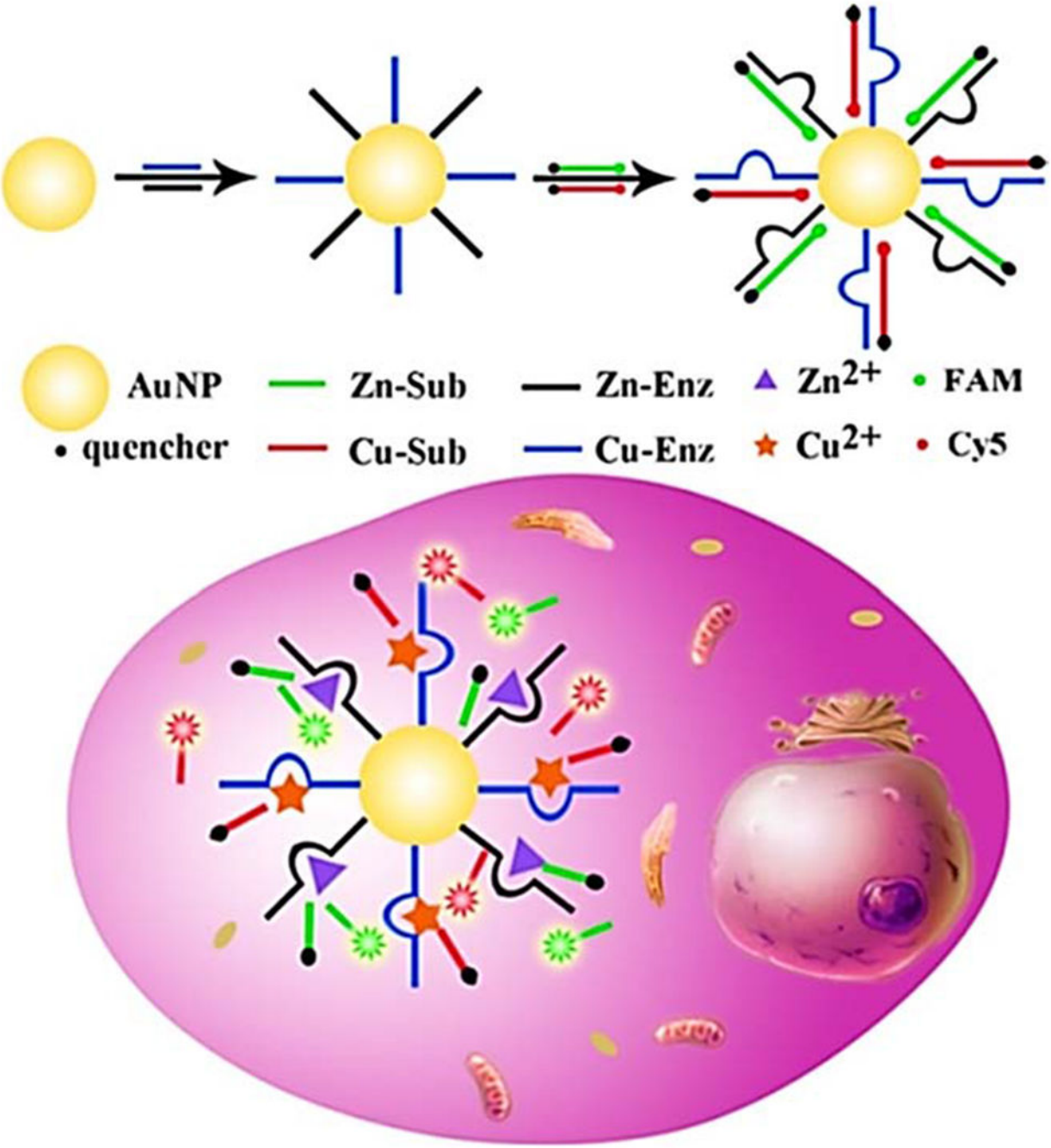

Figure 13.
Detection of intracellular Zn^{2+} and Cu^{2+} ions based on AuNPs-DNAzyme nanoflares[149] with permission.

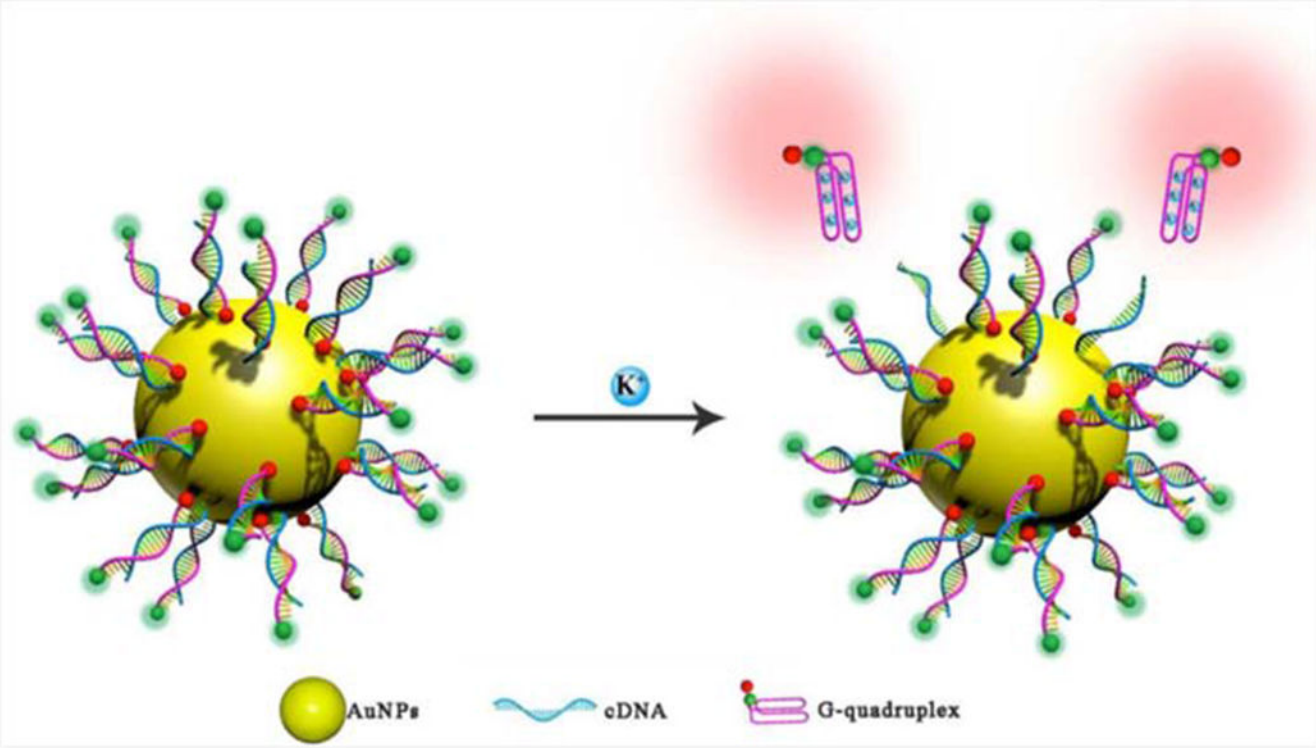

Figure 14.
Interaction of K^+ ions with AuNP-based G-quadrnplex oligonucleotide FRET nanoflares[152] with permission.

Table 1

Recent reports about detection of cancer cells using oligonucleotide.

Nanomaterials	Target	Design	Cell(s) or virus	Dye	LOD	Linear range	Ref.
AuNPs (13 nm)	selenol	Hybridization of AuNPs and Cy5.5 modified peptide chains (Cy5.5-Ser-Asn-Lys-GlyCys)	HepG2[a]	Cy5.5	-	-	104
AuNPs (13 nm)	mRNA	Hybridization of AuNPs and Cy5-modified oligonucleotides	MDA-MB-231[b]	Cy5	-	-	105
AuNPs (13 nm)	mRNA	AuNPs-coupled single-stranded DNA (ssDNA) oligonucleotides	293T[c], HeLa[d], U-373 MG[e], DU145[f], PC3[f], A549[g], BLM[h], RH5[i], RH28[i], RH30[i]	Cy3, Cy5	-	-	106
AgNCs and AuNPs	miRNA	DNA-modified AgNCs [y] and AuNPs	MCF-7[j], HEK239[k]	-	0.4 pM	1 nM-5 μM	107
AuNPs (13 nm)	miRNA (miRNA-21, miRNA-141)	AuNPs functionalized with DNA molecules	HeLa, 22Rv1[l], SMMC-7721[m], LOVE-1[n]	FAM, Cy5	0.9 nM, 1.2 nM	0–25 nM	108
AuNPs	miRNA (miRNA-21, let-7d, miRNA-200b)	Hybrid of tetrahedral DNA nanostructure (TDN) and AuNPs (Au-TDNNs)	HepG2, MCF-7,	-	-	-	109
AuNPs	miRNA-21	duplex specific nuclease (DSN) and bridge DNA-AuNPs	-	-	6.8 aM	10^{-17} -10^{-11} M	110
AuNPs	mRNA	AuNPs functionalized with oligonucleotides	C166[o], SKBR3[p]	Cy5	-	-	111
AuNPs	Human telomerase mRNA	AuNPs and DNA hybridization for construction of “sticky-fare” probe	KB[q], A549	Cy5	-	-	112
AuNPs	mRNA	Commercial spherical nucleic acid modified AuNPs	HeLa	Cy3, Cy5	-	-	113
Core-shell Ag@SiO_2	mRNA, ATP,	Core-shell Ag@SiO_2 and cDNA hybridization	Human blood cells	Cy5	8 μM	0–0.5 mM	114
AuNPs	mRNA	self-assembly of DNA flares hybridization on AuNPs	HeLa	TAMRA[r]	-	-	115
AuNPs (13 nm)	DNA, cocaine,	DNA-modified AuNPs	-	OliGreen,	-	-	116
AuNPs (17 nm)	HIV-DNA	dendrite-modified AuNPs	HIV virus	-	73 pM	150 pM-6 nM	117
AuNPs	Vimentin mRNA	DNA-coated AuNPs	16-HBE[s], MRC-5[t], MEFs[u]	Cy5	-	-	118
AuNPs	ssDNA, dsDNA	DNA probes labeled with various fluorophores	-	FAM[v], ROX[w]	330 pM	1.0 nM–500 nM	119
gold nanodots (AuNDs)	Sulfide ions	MTT[x] capped AuNDs	-	-	2 nM	870 nM-16 μM	120

[a] Human liver carcinoma cells.

[b] Human breast cancer cells.

[c] Human embryonic kidney cells.

[d] Human cervical cancer cells.

[e] Human astrocytoma cells).

[f] Human prostate cancer cells.

[g] Human lung cancer cells.

[h] Human melanoma cells.

[i] Human rhabdomyosarcoma cells.

[j] Human breast cancer cell.

[k] Normal human embryonic kidney cell.

[l] Human prostate cancer cells line.

[m] Human hepatocyte cell.

[n] Human colon cancer cell.

[o] Mouse endothelial cells.

[p] Human breast cancer cells.

[q] Keratin-forming tumor cell of HeLa.

[r] 5-Carboxytetramethylrhodamine.

[s] 16-Human bronchial epithelial cells.

[t] Human fetal lung fibroblast.

[u] Primary mouse embryonic fibroblasts.

[v] 6-Carboxyfluorescein.

[w] Carboxy-X-rhodamine.

[x] 6-Mercapto-s-triazolo(4,3-b)-s-tetrazine.

[y] Ag nanoclusters.