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REVIEW ARTICLE

Fluorescence for biological logic gates

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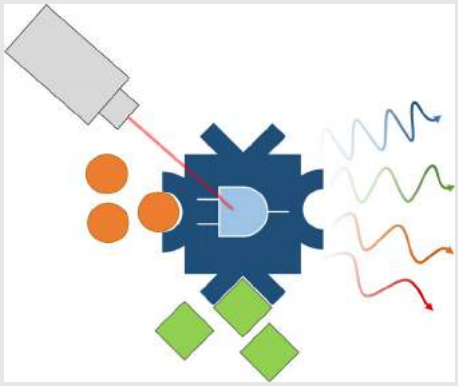
Abstract



Biological logic gates are smart probes able to respond to biological conditions in behaviors similar to computer logic gates, and they pose a promising challenge for modern medicine. Researchers are creating many kinds of smart nanostructures that can respond to various biological parameters such as pH, ion presence, and enzyme activity. Each of these conditions alone might be interesting in a biological sense, but their interactions are what define specific disease conditions. Researchers over the past few decades have developed a plethora of stimuli-responsive nanodevices, from activatable fluorescent probes to DNA origami nanomachines, many explicitly defining logic operations. Whereas many smart configurations have been explored, in this review we focus on logic operations actuated through fluorescent signals. We discuss the applicability of fluorescence as a means of logic gate implementation, and consider the use of both fluorescence intensity as well as fluorescence lifetime.

KEYWORDS

biological logic gates, FLIM, fluorescence, molecular logic gates, reactive probes, smart probes

1 | INTRODUCTION

The ability of nanoparticles to achieve targeted delivery and biological stimulus responsiveness is a field that has been widely explored in the past few decades [1–6]. Such particles are considered "smart" because they are able to only respond to predefined environmental changes relevant for biological conditions of interest such as lowered pH or heightened enzyme activity [7–12]. Many labs are researching such constructs due both to the versatility of nanomaterials and to various unmet clinical needs. For example, both targeting and conditional therapeutic action are able to greatly reduce the side-effects caused by systemic delivery and thus the overall risks to patients [13]. As a foresight for interaction between various types of intelligent nanoparticles, researchers have labeled their advancements molecular logic gates, biological switches, or even predecessors for biocomputing [10, 14].

Abbreviations: AD, anisotropy decay; CD, carbon dot; DOX, doxorubicin; DR, diffusion reflection; EPR, enhanced permeability and retention; FI, fluorescence intensity; FLIM, fluorescence lifetime imaging microscopy; FLT, fluorescence lifetime; FRET, Förster resonance energy transfer; GNP, gold nanoparticle; GSH, glutathione; MEF, metal enhanced fluorescence; MMP, matrix metalloproteanase; MSN, mesoporous silica nanoparticle; NIR, near-infrared; PEG, poly ethylene glycol; QD, quantum dot.

Biological logic gates in the literature often present a wide variety of options for both inputs and outputs. Broadly speaking, inputs can be categorized as either external, such as illumination with a laser or ultrasound, internal, such as enzyme cleavage or ion presence, or both, such as pressure. Logic gate outputs can be categorized as either release of cargo, such as a release of an otherwise hidden or inactive drug, or measurable status change, such as a fluorescent signal or nanoparticle aggregation. Because biological conditions have so many parameters involved, leading to many possible inputs and desired outputs, any logic gate development requires first a determination of the target condition or disease that is of interest to be evaluated by logic operations. Following this decision, the main challenge associated with the inputs is to find a useable set of parameters associated with the application of choice, and to have these parameters be as specific as possible for the application so as to avoid false signals or incorrect treatment. The main challenge regarding the outputs requires first a decision as to whether the system is meant for therapy or diagnosis (or both), and then a decision on what output would be most useful for the chosen application. Figure 1 presents some of the most common logic gate truth tables as well as their symbol representations. Whereas computer logic gates typically have inputs/outputs of 0 or 1, biological logic gates are more complex and the inputs and outputs can be many different parameters. In addition, researchers must take into account factors such as reversibility of the system and its ability to function autonomously or with external guidance.

The concept of biological logic gates did not start overnight, and there are many papers related to the field. Several reviews explore the concept of stimuli-responsive nanoparticles, which are able to provide more efficient and specific drug delivery based on biomolecules or enzymes present in certain medically relevant situations [3, 11, 15–18], and there are also reviews on such smart devices based on DNA origami [19]. Some reviews discuss fluorescent probes activated by certain biological conditions [20–23], and other reviews explicitly refer to logic devices or operations [8, 9, 12, 14]. In the current review, we aim to focus on the use of fluorescence for defining logic gate operations, discussing its applicability for logic gate evaluation and exploring its uses. Section 2 discusses possible choices of output and Section 3 modes of action taken by logic gates, both sections with the goal of setting the stage for a proper discussion. Section 4 covers specific examples of logic gates from different fields more extensively. Finally, Section 5 concludes and

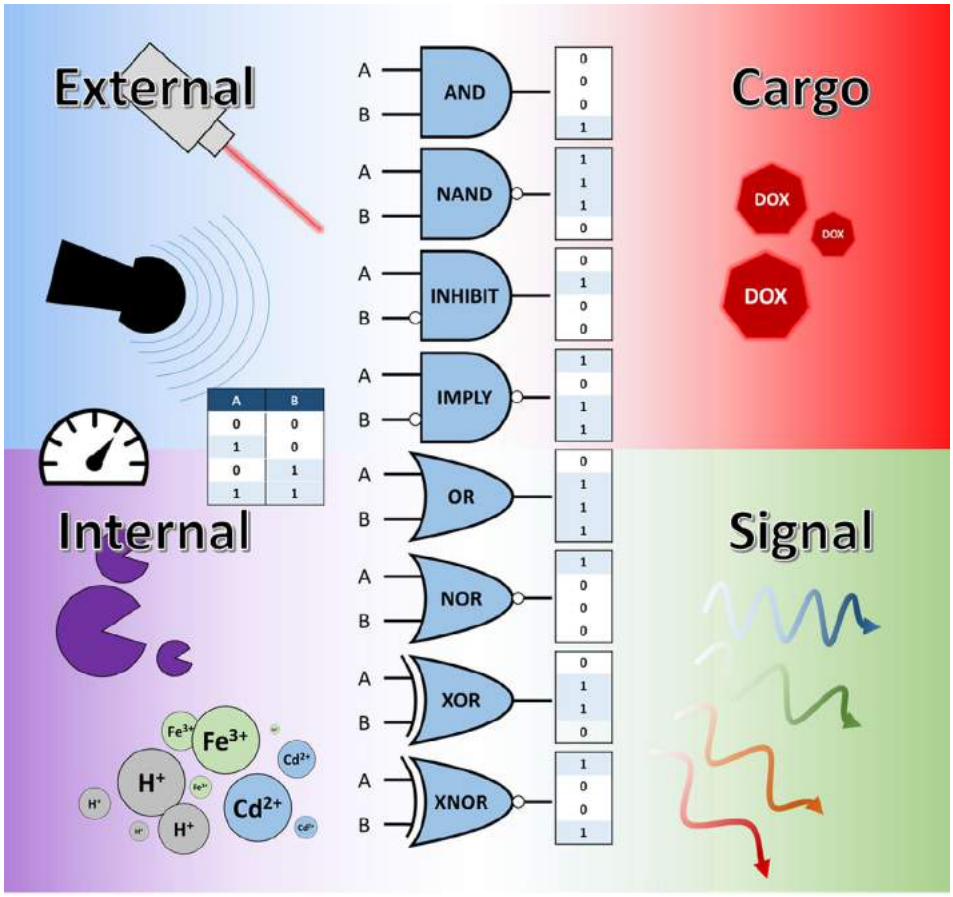


FIGURE 1 Biological logic gate inputs and outputs, truth tables, and graphical representations. A and B, Inputs into the gate, where a 1 indicates the presence of the input (blue-filled table areas) and 0 a lack thereof (white-filled). In biological logic gates, these inputs can be external, such as laser or ultrasound, internal, such as enzymes or ions, or both, such as pressure. Outputs for biological logic gates can be a release of cargo, such as a therapeutic drug, or measurable signal, such as fluorescence, and a response or lack thereof are indicated by 1 and 0 respectively

wraps up the discussions with the potential of such smart particles and their future applicability.

2 | CHOICE OF OUTPUT

Whereas computer logic gates simply translate a combination of electrical input signals into an electrical signal output, biological logic gates are not as straightforward, and their output is usually completely different. The designers must choose in advance the means by which their logic gate will indicate the presence or lack of inputs—they must determine what "0" and "1" mean for the output. In this review, we will discuss three main types of logic gate outputs. One type is the therapeutic effect of the gate—using live animals or cells to see that the logic gates activate only in a tumor site or around specific cells. For example, a set of biological factors work through the logic gate to release chemotherapeutic drugs to shrink a tumor. Although the direct output is the drug, the researchers evaluate the effect by considering how effectively the tumor shrank—the therapeutic effect. Such "therapeutic" logic gates can just as easily measure cell viability, animal death rate, and similar effects that indicate how effective a treatment is. The other two responses we will be examining are fluorescent, and we have split these options into responses based only on changes in fluorescence intensity (FI) (including a change in spectra), and those based on changes to the fluorescence lifetime (FLT). FI logic gates are much more common than FLT gates, but the FLT provides many benefits over traditional fluorescence imaging. Figure 2 presents examples of these three detection methods.

2.1 | Therapeutic effect

The ultimate goal of a biological computer will be a therapeutic one—it would be able to detect and treat diseases appropriately. As such, it is natural to consider biological logic gates that would have a therapeutic outcome by themselves. Since the therapeutic potential of such

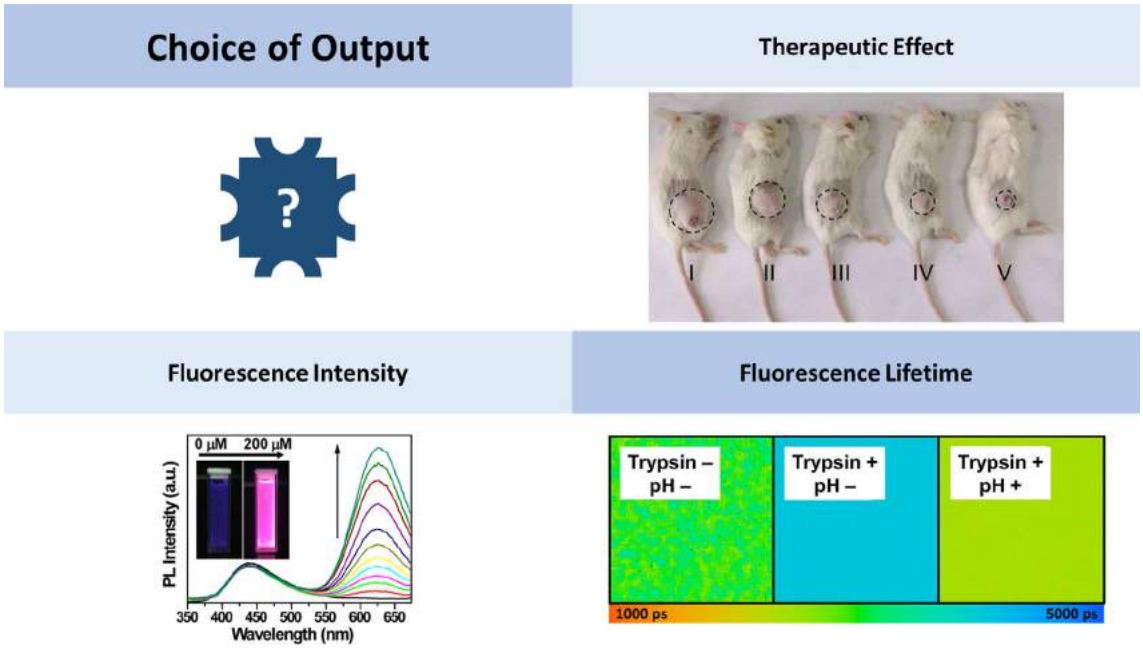


FIGURE 2 Different means of detecting, and thus defining, logic gate outputs. Evaluation of the therapeutic effect of the smart agents can involve placing the nano-agents inside living animals and seeing a therapeutic response due to the correct conditions meeting the agents' input requirements (Reproduced with permission from Chen et al. [24]. 2016, Elsevier). In this image are shown the tumor size variations in mice when provided with varying combinations of reactive elements, and a combined release of therapeutic agents resulted in the smallest tumor volume. Fluorescence intensity (FI) changes can be seen by an increase in the fluorescence signal and/or changes to the fluorescent spectrum as a function of input presence (Reproduced with permission from Niu et al. [25]. 2016, Elsevier). This spectra and photograph reveal how a rising concentration of ascorbic acid increases the observed FI significantly. The fluorescence lifetime (FLT) can also change based on different input combinations, and detected using FLT imaging microscopy (Reproduced with permission from Barnoy et al. [26]. 2019, American Chemical Society). The different input combinations of trypsin and pH can be seen here to result in noticeably different FLT, as can be seen by the change in color of the different panels, since FLIM images create images colored based on the measured FLT

systems is always a core driving force for biological logic gates, we discuss these responses in this review. When talking about a therapeutic response in these studies, we refer to the ability of the logic system to respond to and treat disease conditions either in cells or in animals. For example, this could refer to a probe that knows to release doxorubicin (DOX) only in the presence of cancer cells, whereupon a decrease in cell viability is observed [27], or to a protein able to attach to cell membranes and detectably form pores under predetermined conditions [28]. Alternatively, it could refer to vehicles that are administered to living, tumor-bearing mice and the specific conditions of the tumor environment release proapoptotic agents and the tumor notably shrinks with limited side-effects [24]. When naturally fluorescent substances such as DOX are used, these therapeutic smart materials can be tracked by fluorescence, and researchers often do so [29]. However, the main concern of these studies is still the therapeutic potential of smart devices and so in this review we classify them under this output choice rather than fluorescence. Currently, biological logic gates have still not reached a point that they are used in human beings, and are composed of relatively simple operations—there is still a long way to go to reach biological computers inside people.

2.2 | Fluorescence intensity

Fluorescence imaging has been in use for biological imaging for a long time due to various reasons. It is a relatively easy imaging modality, commonly available, it uses non-ionizing radiation so that it is safe for most living biological tissues, it can provide extremely high-resolution images showing processes even on a subcellular level, and it is able to provide very low background images due to the natural Stokes shift between the excitation light source and emission wavelengths. Even the generally considered drawback of poor penetration into thicker tissue can be addressed by using near-infrared (NIR) fluorescent compounds. Whenever researchers are interested in viewing a biological process in action or wish to discover a mechanism of action, fluorescence is usually one of the first options [22, 30]. As such, it is no surprise that many biological logic gates have made use of this tool.

For the purposes of this review, we are referring here to FI imaging, where the detected signal is usually measured in arbitrary units describing how many photons were able to reach a detector. An example AND gate could be a system where a fluorophore is quenched unless two inputs are present, upon which a fluorescent signal of much stronger intensity is observed. Nikitin et al. made a system of various logic operations, where a "true" or "false" response was decided by whether the FI was greater than a certain cut-off [31]. Zhu et al. described their logic operation by changes to the fluorescence spectra in different situations, observing whether one or two peaks could be seen based on input situations [32]. Bui et al. determined gate output based on the presence or lack of Förster resonance energy transfer (FRET) signals [33]. Intensity, spectra, and FRET techniques are all viable FI determination schemes.

2.3 | Fluorescence lifetime

FLT imaging microscopy (FLIM) is a powerful diagnostic tool with characteristics that make it extremely useful for determining biological logic gate outputs. The FLT of a fluorophore is a statistical measure of the number of time electrons of the molecule remain in the excited state and is inherent to the fluorophore. This property does not depend on the fluorophore concentration or lighting conditions the way FI does. The implication for biological samples is that we do not need excessive amounts of analyte to reach a region of interest, simply enough to produce a detectable signal. That being said, although the FLT is inherent to any particular fluorophore, it is still subject to change based on the environment. The result is a quantitative parameter able to distinguish between different levels of activation for a smart probe. The susceptibility to environment combined with immunity to concentration and lighting limitations makes FLIM a great candidate for functional imaging able to communicate in vivo environmental changes to an outside observer, and an improvement over pure FI measurements. Some examples of the use of FLIM for detecting biologically relevant situations are in the use of fluorescein diacetate to detect solution pH [34], Bodipy molecular rotors to detect viscosity [35], or ion-binding molecules to detect intracellular mercury [36].

For biological logic gates, the design of a fluorescent probe will determine whether it would be applicable for FLIM or only FI—not all probes with initial quenching necessarily result in a FLT-detectable agent. To understand this concept, we present the equation for the FLT:

$$\tau = \frac{1}{\Gamma_{\text{radiative}} + K_{\text{non-radiative}}}$$

In this equation, τ is the FLT of the molecule, $\Gamma_{\text{radiative}}$ is the radiative decay rate, and $K_{\text{non-radiative}}$ is the non-radiative decay rate of electrons from the excited to the ground state. Non-radiative processes could include heat or movement of the molecule. When a fluorophore

is bound to metallic nanoparticles, for example, there are more radiative and non-radiative decay paths for a given electron's energy to take (to the metal), and the result is that the FLT detected for the molecule is shorter than unbound (the denominator in the above equation is larger) [37]. Gold nanoparticles (GNPs) have been shown to affect the FLT of bound fluorophores and affect their subsequent imaging in biological tissues [38, 39]. The freedom of movement of the fluorescent molecule itself can also affect the FLT, meaning that a molecule made rigid would have fewer non-radiative processes and so a longer FLT [36]. In other situations, quenching might be caused not by changes in the radiative and non-radiative decay rates, but rather because the entire fluorescence photon is absorbed by the quencher. In these cases, we observe static or contact quenching, which means that the ground state of the complex changes from that of the unaffected fluorophore, but the decay rates are the same. The implication is that the FLT does not change when the molecule is quenched in this manner—the above equation has no changes to it [40, 41]. For these systems, FLIM cannot be relevant for logic gate detection.

3 | MODES OF ACTION

Biological logic gates can be categorized based on the manner in which they function. Figure 3 summarizes these options, and this section further expands on the idea. For the purposes of this review, we have characterized biological logic gates as single input responders, multiple input responders, sequential activators, reversible systems, and/or interacting components. Note that not all of these characterizations are necessarily exclusive, so a sequential activator could, in theory, respond to multiple inputs and also be reversible.

3.1 | Single input responders

Single stimulus responders are the basic building blocks of biological computations. In order to have more complex operations, the basic operations need to be established. This label refers to a group of nanoparticles capable of reacting to or detecting only one particular type of molecule, enzyme, substance, or environmental condition. A single response of YES/NOT—whether the substance is present in the current situation or not—is not particularly interesting to the field of biocomputing on its own, but serves as the basis for future biological computing if multiple such components are combined. Due to the relative simplicity of designing and assembling these systems, many labs and researchers have developed such nanoparticles. Gu et al. created polymer nanoparticles that release insulin as a response to surrounding glucose concentrations [42]. Tian et al. bound carbon dots (CDs) to silver nanoparticles, and detected a fluorescent response in the presence of an antioxidant presence such as ascorbic acid [43]. Many other such examples exist. For fluorescent logic gates, such agents can respond to input presence with quenched fluorescence that is restored [44], shifts in fluorescent spectra

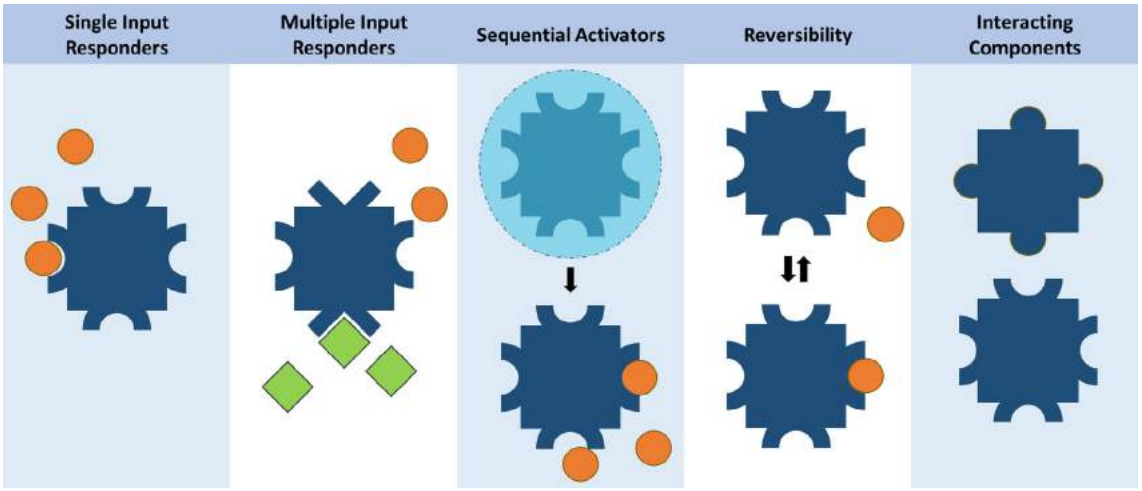


FIGURE 3 Different modes of action that biological logic gates may take. Single input responders respond to only a single input (although it is possible for other similar compounds to generate the same effect). Multiple input responders require more than one input to generate an output, thus making their activation more complex. Sequential activators may respond to either one or more inputs, but there are different stages of activation that must happen in a predetermined order. Reversible systems are able to "deactivate", or switch between "on" and "off" states. Interacting components are systems of multiple units that are able to affect each other

[45], metal enhanced fluorescence (MEF) [43], and/or change in the FLT [34].

3.2 | Multiple input responders

More complex than the single stimulus responders, multiple input responders require more than one factor for their response. Multiple input responders can be thought of as combinations of the single input responders, but with a response dependent on all inputs [31]. Systems like these are able to specifically target certain conditions because various parameters may be present throughout the body at any time, but a combination of them might only occur in disease. For example, a simple PET scan would not be able to distinguish between inflammation and cancer due to the increased glucose uptake in both situations [46]. Meanwhile, a system that would fluoresce only when both matrix metalloproteanase (MMP) enzymes are active and low pH could more accurately detect cancer [47]. When the probes can respond to multiple inputs, it is possible to design the more complex logic operations of AND, OR, NAND, NOR, XOR, XNOR, and others. The possible responses in fluorescent logic gates are the same as those found in the single input responders.

3.3 | Sequential activators

Sequential activators may require multiple inputs or could even function with a single input of varying levels. Whether one of varying degrees or multiple, in these systems the inputs necessarily have to take place in a particular order and this order is crucial for proper functioning. Thus, although multiple inputs are involved, the process is better described as an IF/THEN situation rather than directly multi-input gates such as AND and OR. Nevertheless, such sequential systems are just as important to the concept of biological computing as IF/THEN loops are to traditional programming. The review of Pacardo et al. takes a look at programmable nanocarriers for cancer treatments, with an emphasis on synergistic and sequential drug delivery systems [48]. Such treatments can autonomously target and treat specific conditions because of their situation-dependent behavior.

Usually, sequential activators are cases where the nanoparticulate system has some form of initial shielding feature that allows for biocompatibility and targeting to tumor microenvironments. Upon reaching such locations, low pH leads to the exposure of the inner structure, which is able to then act only in that particular area of the body that was able to remove the shielding. For example, Wang et al. developed nanoparticles sensitive to the acidity of tumor microenvironments, and following NIR irradiation fully activates a therapeutic drug [49]. Ruan et al. used GNPs that have a MMP2-sensitive coating so that the particles shrink in tumor sites and then release DOX rapidly by the surrounding acidic pH [50]. Li et al. described clustered particles that disaggregate in tumor acidity, and then the released smaller units easily enter cells to release cisplatin [51].

Subsequent steps can also be achieved such as where the first steps facilitate entry into cells, and then other actions occur only within the actual cells of interest. The Zhang lab used complexes with a detachable shielding surface of charge-switchable poly ethylene glycol (PEG) chains. Tumor acidity removed the PEG shield, exposing folate to allow for easy cell uptake. In the cell cytoplasm, the relative abundance of glutathione (GSH) cleaved certain disulfide bonds to release a proapoptotic peptide and gene and thus kill the cell [24]. In the same year, the Zhang lab also described a system consisting of DOX on peptide chains with pH-sensitive hydrazine bonds. The positively charged peptides are able to enter cells, and then the hydrazine breaks inside endosomes to release the DOX while a killing gene is released in the cytoplasm by GSH disulfide bond cleavage [52]. Chen et al. used siRNA-containing NPs, which respond to surrounding low pH followed with redox by GSH to release siRNA to kill the cancer cells [53]. Similarly, Yan et al. arranged prodrugs in micelle form to reach low pH sites, where the micelles break down, and then GSH activates the drugs within the cell cytoplasm [54]. Finally, Han et al. created nanovectors that accumulate in tumor sites by the enhanced permeability and retention (EPR) effect, whereupon MMP-9 degrades a PEG corona to expose an agent that allows for internalization. Following that, cathepsin B in the lysosomes releases gemcitabine to kill the cells [55].

Mesoporous silica nanoparticles (MSNs) serve as efficient sequential activators due to their ability to both store and be coated. The MSNs described by Xiao et al. easily enter cells in low pH because of their coating, and then the redox reaction occurring around GSH releases drugs from the pores within the cells [56]. Han et al. coated such NPs with PEG for passive targeting, but low pH exposes surface ligands to facilitate cell uptake, and even lower pH in endosomes or lysosomes releases DOX [57]. As a final example, Liu et al. fabricated MSNs with a zwitterionic coating that becomes entirely positively charged in the tumor environment, allowing for cell entry where quantum dots (QDs) and DOX are released [58].

The work of Yan et al. provides a good example of how fluorescence can make for a valuable tool in the

detection of sequential activators [54]. As described above, these researchers placed GSH-sensitive prodrugs inside pH-sensitive micelles. Each step—the breaking of the micelles and the activation of the drugs—could be detected individually since the micelle dissociation led to 830 nm fluorescence and subsequent GSH activation shifted this fluorescence peak to 630 nm. This ability to provide a different fluorescent response in each step in the sequence can be incredibly useful for diagnostic purposes, as such smart probes could have their action process tracked to make sure that the activation steps occur in the correct place and time.

3.4 | Reversibility

Systems that are reversible are interesting for biological computing because they allow for a reevaluation process. Reversibility for biological logic gates means that any particle could perform the computation for which it was designed multiple times without having to constantly insert more doses into the patient. Any particular nanoparticle that had already activated by a condition could revert back to the previous state to either indicate that the disease has been defeated or to stop releasing potentially toxic chemicals. Systems that respond to cleaving enzymes cannot be reversible [59], but shape altering protein reactions [60], pH sensitivity [61], and ion-binding molecules [62] might very well be reversible. For fluorescent logic gates, reversibility means that the fluorescent signal response can be reset, such as back to a quenched state after fluorescing if the input is no longer present.

3.5 | Interaction between components

Although much more rare in the literature, interacting components are mentioned in this review due to their promising potential for biological computing. Whereas a single type of nanoparticle that can detect or treat a specific condition can be very beneficial, nanoparticles that could affect others could greatly improve clinical applications. One kind of effector particle could be used for multiple conditions, while secondary particles that require the effector could then further specify the scope of the entire system. It is these interacting components that would eventually lead to a true biological computer. Amir et al. describe interacting DNA origami nanorobots, where an effector robot opens in certain conditions, and its contents are able to open or close other robots to produce fluorescent signals [63]. As with the other modes of action, a fluorescent signal provides a useful means of evaluating the efficacy of the system.

4 | DIFFERENT KINDS OF LOGIC GATES

In this section, we will discuss several logic gates that were developed for different types of applications. We have split the studies based on their choice of output for determining logic gate activity, and so we present logic gates primarily for therapeutic effect, logic gates mainly fluorescent and described by FI, and logic gates designed for FLIM. Several of these examples have been mentioned above, but they are organized and grouped with similar studies.

4.1 | Intelligent therapeutic solutions, using FI for detection

Many labs have worked on intelligent therapeutic agents, able to treat certain diseases in a more specific manner than traditional medicine. In order to track the fate of their constructs, they sometimes use FI to detect the mechanisms of action and progress. While these systems are not necessarily described as "biological logic gates," they do offer an intelligent solution that is specific to certain biological situations. In this review, we are only looking at these therapeutic response studies that have at least made some use of fluorescence.

One direction of study has been relatively large constructs able to target tumor regions, where the size shrinks, deeper tumor accessibility is attained, and finally, therapeutic agents are released to treat the tumor [50]. Such studies have also made use of overexpressed proteins found in certain cancers for further, better targeting [64], and also ligands to increase cell uptake [49]. Some researchers also added steps past the tumor targeting, to also allow for endosomal escape and further enzyme degradation intracellularly to release therapeutic drugs [24, 27, 51, 52, 54, 55, 57, 58]. Others have used similar techniques to release siRNA and affect genes [53]. Others yet employed GNPs and other materials to allow for PTT as treatment [47]. Zhang et al. combined several blocks and developed a library of responsive polymers to create plug-and-play responsive nanocarriers able to treat cancer cells both in vitro and in vivo as a response to variable inputs [33]. Another avenue has been the development of proteins able to associate into a cell membrane and alter it as a response to environmental conditions [65]. Many of these studies make use of the EPR effect found around tumors, the slightly reduced pH found in tumor regions or in organelles, and intracellular conditions such as heightened biothiol content. Multiple enzymes were used to affect the probes, such as the MMP enzymes found in increased amounts around tumors.

TABLE 1 Intelligent therapeutic solutions

Source	Logic gate	Inputs	Outputs	Gate evaluation	Reversible?
[50]	Sequential activator	EPR, MMP2, low pH	Therapeutic effect	Therapeutic effect, FI	No
[64]	Sequential activator	EPR, LRP, MMP2	Therapeutic effect	Therapeutic effect, FI	No
[52]	Sequential activator, OR	EPR, low pH, GSH	Therapeutic effect	Therapeutic effect, FI	No
[51]	Sequential activator	EPR, low pH, reduction	Therapeutic effect	Therapeutic effect, FI	No
[57]	Sequential activator	EPR, low pH, galactose	Therapeutic effect	Therapeutic effect, FI	No
[24]	Sequential activator, OR	EPR, low pH, folate, GSH	Therapeutic effect	Therapeutic effect	No
[58]	Sequential activator	EPR, low pH, esterases	Therapeutic effect	Therapeutic effect, FI	No
[47]	Sequential activator	MMP, low pH	Therapeutic effect	Therapeutic effect, FI	Partially
[53]	Sequential activator	EGFR, low pH, GSH	Therapeutic effect	Therapeutic effect	No
[27]	Sequential activator	Low pH, GSH	Therapeutic effect	Therapeutic effect, FI	No
[55]	Sequential activator	EPR, MMP9, cathepsin B	Therapeutic effect	Therapeutic effect, FI	No
[49]	Sequential activator	EPR, low pH	Therapeutic effect	Therapeutic effect, FI	No
[54]	Sequential activator	Low pH, GSH	Therapeutic effect	Therapeutic effect, FI	No
[28]	Sequential activator, OR, AND	Low pH, GSH, several others	Therapeutic effect	Therapeutic effect, FI	No
[65]	AND, OR-AND	Low pH, inhibitor, MMP9	Therapeutic effect	Therapeutic effect, FI	Yes

Note: The logic gates discussed in Section 4.1 are ordered by year of publication in this table.

Table 1 summarizes some of the important aspects of the studies considered for this section, and especially how they relate to the field of biological logic gates. It can be noted that nearly all of these examples described sequential activators, and a lot were tested in vivo. Most were not reversible.

4.2 | Logic gates using FI

Several labs have explored the option of using fluorescent signals to determine logic operations. The systems of interest here are those based on multiple orthogonal and biologically relevant inputs that are able to activate fluorescence in such a manner as to perform logic functions such as AND. Some of these projects had the added benefit of being reversible.

De Silva et al. were among the first to describe logic operations by means of molecules, and used fluorescence to do so [66]. Others continued similar work to have combinations of ions or other biologically relevant molecules affect the FI [25, 32, 43, 60, 62, 67–77], sometimes in very versatile settings to allow for easy manipulation of the system to create any desired logic gate [31, 78]. Another direction has been the use of enzymes to affect the FI in logic operations [33, 79]. Uchiyama et al. were the first to create a fluorescent logic gate using a non-chemical input-temperature [61]. MSNs allowed researchers to make use of several release elements in a logic operation to release fluorescent cargo [56, 80]. Remón et al. created the first realization of reversible logic operation—not in the chemical sense, but in the logic sense. This reversibility means that it is possible to identify which input combination was used since each output maps directly to one input set, and the logic gate is called a controlled-NOT (CNOT) or Feynman gate [81]. Douglas et al. constructed DNA-origami nanorobots able to respond to different cues to expose fluorescent payloads, showing various logic operations in so doing [82]. This work was later continued by Amir et al. to display such nanorobot interactions to achieve even more complex operations, even inside living cockroaches [63]. Other DNA-based studies have used a CRISPR system to behave as an AND gate [83] or incorporated into cell membranes to detect dangerous cells by their surfaces [84].

Table 2 summarizes information from the studies presented in this section. An interesting note that can be seen from this table is that there are many different kinds of logic operations achieved, and a few of the operations were fully reversible. The systems described in this section only made use of FI, and either ignored or discarded FLT information. For example, Liao et al. measured only small FLT changes and concluded that the effect is caused by static quenching [72], and similarly with Zhai et al. [70], Zou et al. [71], Ghorai et al. [73], Zhang et al. [76], and Li et al. [62] Although *Yuan* et al. did measure the FLT and detected a significant decrease in the presence of glyphosate, they merely stated that such a reduction is a good indication of FRET and did not use the measure further [44]. Both Remón et al. and Bai et al. claimed to have measured FLT, but made no

TABLE 2 Logic gates described by FI

Source	Logic gate	Inputs	Outputs	Gate evaluation	Reversible?
[66]	AND	Hydrogen and sodium ions	FI change	FI	No
[60]	YES	Calcium ions	FI change	FI	Yes
[61]	AND	Temperature, pH	FI change	FI	Yes
[81]	CNOT	Hydrogen ions and dihydrogen phosphate anions	FI change	FI	Yes
[80]	AND	Light, pH	FI change	FI	No
[82]	Several	Several	FI change	FI	No
[56]	Sequential activator	EPR, low pH, GSH	FI change	FI, therapeutic effect	No
[78]	Several	Several	FI change	FI	No
[63]	Several	Several, interaction	FI change	FI	Yes
[67]	IMPLY	Copper ions, pyrophosphate	FI change	FI	No
[31]	Several	Chloramphenicol, fluorescein, interaction	FI, exposed receptor	FI	No
[79]	AND	Glyocisdase, exopeptidase	FI change	FI	No
[32]	Keypad lock, NOR-AND	Auramine O, pH	Fluorescence spectrum changes	Fluorescent spectroscopy	No
[25]	Sequential activator	Cadmium ions, ascorbic acid	FI change	FI	No
[43]	IMPLY	Ascorbic acid, iron ions	FI change	FI	No
[68]	OR	pH, excitation	Excitation change	FI	Yes
[69]	INHIBIT	Mercury and iodine ions	FI change	FI	Yes
[70]	IMPLY	GSH, $S_2O_8^{2-}$	FI change	FI	No
[71]	Several and combinations	Zinc ions, copper ions, sulfur ions, pH	FI change	FI	No
[72]	IMPLY	Mercury ions, GSH	FI change	FI	Yes
[73]	AND-XOR	Zinc ions, copper ions	FI change	FI	Yes
[33]	OR	Trypsin, chymotrypsin	FI and spectra changes	FI, FRET	No
[74]	AND	Homocysteine, peroxynitrite	FI change	FI	No
[75]	Several	Several cations and anions	FI and spectra changes	FI	Yes
[76]	Yes	Chlorpromazine hydrochloride	FI change	FI	No
[83]	AND	DNA strands	FI change	FI	No
[84]	AND	Membrane proteins	FI change	FI	No
[77]	IMPLY, NOR	Varying pH, copper ions	FI and MR changes	FI and MRI	Yes
[62]	XNOR	Iron and fluoride ions	FI change	FI	Yes

Note: The logic gates discussed in Section 4.2 are ordered by year of publication in this table.

mention of the values [69, 81]. Fang et al. measured the FLT for CD characterization, but not after introduction of analytes [77].

4.3 | Logic gates using FLT

As discussed previously, the FLT provides a great tool for biological logic gate applications, and could greatly enhance other fluorescent indicator systems when it is applicable for detecting different situations. There are noticeably fewer examples of such FLT-based systems in the literature.

Several materials have been created to respond with a changed FLT to a single condition, such as with temperature [85] or with viscosity [35, 86], and some to respond to single chemical inputs, such as glyphosate [44], pH [34], or various ions [87]. Others developed nanostructures that respond by FLT or a combination of FI and FLT to describe various logic operations following exposure to multiple inputs [26, 88–91]. Some researchers did create probes responding to various

TABLE 3 Logic gates described by FLT

Source	Logic gate	Inputs	Outputs	Gate evaluation	Reversible?
[85]	YES	Temperature	FLT change	FLIM	Yes
[35]	YES	Viscosity	FLT change	FLIM	Yes
[88]	AND, Sequential activator	Low pH, esterases	Therapeutic effect, FLT and FI change	Therapeutic effect, FI, FLIM	No
[92]	Several	Iron ions, fluoride ions	FI and spectra change	FI, FLIM	Yes
[86]	YES	Viscosity	FLT and FI change	FLIM, FI	Yes
[89]	AND	Iron ions, pH	FLT and FI change	FLIM, FI	No
[93]	OR, Keypad lock	Zinc ions, aluminum ions	FI change	FI, FLIM	Yes
[94]	INHIBIT	Magnesium ions, fluoride ions	FI change	FI, FLIM	Yes
[95]	NOT, OR	Aluminum ions, mercury ions	FI spectra change	FI, FLIM	No
[44]	YES	Glyphosate	FLT and FI change	FLIM, FI	No
[34]	YES	High pH	FLT change	FLIM	No
[90]	Several	High pH, caspase 3	FLT and FI change	FLIM, FI	No
[91]	AND	Low pH, iron ions	FLT and FI change	FLIM, FI	No
[26]	Several	Low pH, trypsin	FLT change	FLIM	Partially
[96]	AND	Copper ions, GSH	FI change	FI, FLIM	Yes
[97]	Keypad lock	Various metal ions	FI change	FI, FLIM	Yes
[36]	INHIBIT	Mercury ions, sulfide ions	FI change	FI, FLIM	Yes
[98]	AND, tandem AND	Ciprofloxacin, 370 nm excitation	FI change	FI, FLIM	No
[87]	YES	Iodide ions	FLT, FI and spectra changes	FI, FLIM	No

Note: The logic gates discussed in Section 4.3 are ordered by year of publication in this table.

chemical inputs and used the FLT to differentiate situations, but ultimately relied only on the FI for logic gate determination [36, 92–97]. Wang et al. developed a system that uses two AND gates in tandem to achieve three tiers of ultimate output [98]. As with others, this tandem system was defined with FI outputs, but was also shown to respond accordingly in FLT.

Table 3 summarizes information about the studies presented in this section. Like with the FI logic gates, these FLT gates have shown the potential to achieve complicated logic operations and some reversibility. A fair number of researchers all measured the FLT and even measured different values for different logic states, however, they did not use these values for their logic gates [36, 92–98]. They have been included in this section because the FLT was still shown to be a viable option.

5 | CONCLUSIONS

5.1 | Other possible directions

There exist a few avenues that can present routes for further studies into fluorescent logic gates. One option is the use of metallic nanoparticles, which can significantly affect the fluorescence of fluorophores in their vicinity. GNPs, in particular, are interesting due to their non-toxicity and biocompatibility [99], and hence will be the main subject of discussion here. GNPs offer many useful characteristics for biological logic gates, among them tunable optical properties such as scattering characteristics [100, 101] and easy surface modification [102, 103]. Whereas GNPs by themselves can serve as efficient diagnostic [104–106] and therapeutic [107, 108] agents due to their high density for CT detection [46] and susceptibility to localized heating by infrared irradiation [109, 110], their easily adjustable surface modifications provide them with programmable interactions with their surroundings all the while possessing the ability to be monitored by CT, diffusion reflection (DR), or similar techniques [38, 111, 112]. Peptide-functionalized GNPs have long been known for their potential for therapeutics and delivery [113]. Through intelligent GNP coatings, researchers have developed logic gates based on GNP aggregation/dissociation, which can even be detected by eye [114, 115]. More directly connected to this review, however, the various options for GNP coatings also make them very capable vehicles for fluorescent compounds.

Due to the GNPs' optical properties, however, unlike the typically limited interaction between vehicle and cargo, GNPs interact with nearby fluorophores in a synergistic manner [37]. The GNPs are able to significantly affect the fluorescence of molecules found in their near-field and so possibly cause drastic quenching or enhancement, two processes discernible through changes in the FLT and shown to be observable in biological settings. By considering the ability to modify GNP surfaces according to any desired configuration so as to make them responsive to predetermined biological signals, the effect the GNPs have on nearby fluorophores that can be measured by both FI and FLIM, and their natural biocompatibility, it can be easy to imagine GNPs as promising biological logic gate elements.

Another promising direction is that of anisotropy decay (AD). In a similar manner to which the intensity decay provides more useful information compared to the intensity by itself, the AD provides yet another dimension of information [41, 116]. As with the FLT, AD is not sensitive to experimental factors that impede FI measurements [117, 118]. By considering how the anisotropy of a fluorophore changes over time, it is possible to evaluate parameters that affect the spatial orientation of the fluorophore. AD is mainly caused by the rotational diffusion of the fluorophore, but can also be affected by such effects as energy transfer. The result is a restriction in the fluorophore's rotational movement in various situations and subsequent restoration upon conditions being met [119]. AD can be used to evaluate changes in intracellular viscosity [120] changes in proteins associated with the cell membrane [121], or changes in protein structures [119] or antigen-antibody interactions [122]. AD has been used to study the dynamics of various molecules and proteins and interactions within cells [123–125]. As a useful tool for evaluating fluorescent dynamics, AD can easily translate to and greatly support the field of biological logic gates, where fluorescent properties change under conditions of interest.

The use of GNPs and AD are but two examples that have the potential to improve fluorescent biological logic gates. Other options exist, and the field is still in rapid growth and development.

5.2 | Lessons and perspectives

Biological logic gates serve as promising agents for intelligent medical care in the future. Their use has not translated to human subjects yet, but as seen in this review there are many examples in solutions, cells, and living animals. Tables 1-3, all summarize the examples shown in this review of logic gates predominantly for therapeutic purposes, fluorescent logic gates, and logic gates (at least potentially) defined by FLT, respectively. All of these research works provide examples of the biological logic gates being developed for detection by fluorescence, or for therapeutic uses with the potential for fluorescence tracking. It is worthwhile to notice trends in these kinds of logic gates to see how the field is developing, and how it can become more significant for future clinical applications. Likewise, it is worthwhile to discuss how fitting are the tools of FI and FLT to the field of biological logic gates.

A major difference in trends between the kinds of logic gates can be discerned by considering the "Logic Gate" column of each table. It is apparent that nearly all of the therapeutic logic gates presented here are sequential activators, whereas the two kinds of fluorescent ones have a much broader spectrum of possible logic operations by which they detect. There seems to be a gap between the possible diagnostic logic gates developed by researchers, and the logic gates that researchers feel are actually therapeutically applicable—whereas many teams develop probes for multiple concurrent inputs, the ones actually making smart therapeutic tools try those activated in steps. Whereas purely fluorescent systems will not be therapeutic by definition, it is still possible to use the same tools designed for the diagnostic methods in therapeutic situations, such as by the activation of a prodrug instead of the fluorophore. Zhang et al. have shown that the possibility of incorporating more complex gates into sequential activators [28].

Another finding from this review is that biological logic gates based on FLT are rare. Research about materials that respond to at least one input by a change in FI or spectrum are plentiful—the field of activatable fluorescence is a large one [126–128]. Even studies where the FLT notably changes as a response to a single input are not difficult to find [118]. Complex logic operations, which require at least two different inputs and the output is a change in the FLT, however, are a different story. We have mentioned a few such examples in this review, but unlike the other categories, this sample is closer to the whole of such research of which we are aware. Part of the rarity of this topic is a product of the much more common FI instruments readily available compared to FLIM. Another important factor is the issue of the FLT not always being relevant for logic gate switching, due to situations such as static quenching. Whichever reason leads to this limited sample, it is our opinion that there are many opportunities for logic gates to make use of the FLT where it is simply ignored. As mentioned in Section 4.3 and Table 3, there are also quite a few cases where the FLT was shown to have the potential of defining logic gates, but the FI was still ultimately preferred.

As with non-sequential activators for therapeutic logic gates, FLT logic operations are still a developing field for fluorescent logic gates.

With these considerations in mind, it is important to discuss the usefulness of fluorescence techniques for biological logic gates. Two main questions are raised: when is fluorescence useful for biological logic gates, and is FLIM a useful tool for the purpose? Fluorescence, in general, can be extremely useful for the field in general, as it is for any biological imaging where it can provide very high resolution and real-time tracking of the procedure. Whether therapeutic effects are incorporated or not, having a means of efficiently evaluating the progression always benefits diagnostics. However, as with any imaging modality, fluorescence comes with drawbacks. Visible light does not penetrate biological tissue very well, making deep imaging of such probes near to or completely impossible [106]. Optical fibers can be inserted into animals, but in this manner pose a very invasive imaging method. Using NIR light fluorescence does increase penetration depth, but only to the order of a few centimeters. There is currently no ideal manner of deep-tissue live animal fluorescent imaging of any kind. For the situations where fluorescence is a valid choice, however, FLIM has the potential to improve it further. Granted, the situations where the FLT will not change, such as with static quenching, cannot be considered for FLIM, but the situations where the FLT does change can benefit greatly. FLIM provides more quantitative and reliable detection compared to traditional FI. In cases such as logic gates, where we look for clear-cut distinctions between different states, such a number is much more useful, and especially when factoring in possible sources of intensity problems in biological settings that can affect the excitation, emission, or both. Therefore, although situational, we believe that FLIM can provide a very useful tool for biological logic gates and should be considered more frequently in their evaluation.

5.3 | Summary

Smart materials responding to specific biological conditions are the current trend in nanomedicine research, and with good reason—their potential for improved and more personalized medicine. Biological logic gates are the next step, where these stimuli-responsive agents are able to even more specifically identify and treat prespecified situations. Using a behavior similar to computer logic operations, these developing constructs are able to conduct a basic computing at a biological level, and in this manner pave the way for in vivo computers.

This review has covered some trends and accomplishments in recent biological logic gates already creating therapeutic outcomes, and those that use fluorescence to allow for smart diagnostic capabilities. These research activities are groundbreaking, and can lead to significant theranostic advances. Although complex biological logic gates are still not readily available in the clinic, healthcare professionals could greatly benefit from their aid. For the most part there are still only very specific logic gate inputs and outputs available only as proofs of concept, but researchers are working on creating plug-and-play devices to cater any physician's needs based on their disease of interest [33]. There is still a lot of work to do to reach full clinical applicability.

The current coronavirus disease (COVID-19) affecting the world is an example where smart medicine could be very useful. It is still very difficult to detect the disease, not to mention the current lack of direct treatment. Research has already discussed the applicability of smart chemical detection systems for COVID-19 [18], and researchers are developing various biological stimulus-driven mechanisms to aid in the effort against the disease. Logic gate-like devices offer an ideal tool to effectively treat such a new, fast-spreading, and hard-to-detect pandemic, and also such well-known conditions as cancer that still pose great challenges worldwide.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

AUTHOR CONTRIBUTIONS

All three authors were involved in conceptualization, investigation, and writing.

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