Image Processing and Luminescent Probes for Bioimaging Techniques with High Spatial Resolution and High Sensitivity

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Abstract. The balance of microenvironmental factors (including temperature, pH, ROS species, etc.) plays a crucial role in maintaining normal living organisms' normal physiological activities and physiological functions. Therefore, armed with the unique superiorities of high spatial resolution, non-invasion, high sensitivity, real-time monitoring, and simple operation, luminescent imaging technology has been widely used in real-time and accurate monitoring of microenvironmental factors in these organisms to prevent, diagnose and treat related diseases in time. However, due to its optical imaging characteristics, it is also faced with such interference factors as relatively shallow imaging penetration depth, background fluorescence (biological autofluorescence) interference in a complex environment, uncertain probe concentration, and unstable laser power in the imaging process, which are not related to the analyte. As for the problems in imaging, such as the uncertainty of probe concentration and the fluctuation of instrument laser power, the ratio detection, and imaging technology with self-calibration function can effectively avoid these problems. As for background fluorescence interference in imaging, probes with long-life emission can be used in imaging. The long-life luminescence of probes from background fluorescence can be recognized by time-resolved luminescence imaging technology to reduce its impact. This paper briefly introduces and summarizes the relative research of ratio detection and imaging technology and time-resolved luminescence imaging technology.

Keywords: Ratio-luminescent imaging, Time-resolved luminescent imaging, Probes.

1. Introduction

Bioimaging techniques have become one of the essential tools used in cancer investigation, clinical examination, and medical practice. Despite the problems and challenges, bioimaging techniques were booming over the past decades, and their imaging methods and application range become increasingly varied [1]. The present bioimaging techniques can analyze a particular molecule, allowing researchers to study physiological processes qualitatively or even quantitatively in vivo circumstances [2-13].
There are abundant types of bioimaging covering X-ray, positron, photon and acoustic imaging, etc. Imaging spatial resolution is also varied and can be applied at the macro, mesoscopic or microscopic levels. Currently, macro imaging techniques that provide anatomical and physiological information, such as Ultrasound Imaging (US), Computed Tomography Imaging (CT) and Magnetic Resonance Imaging (MRI), are widely used in clinical and preclinical studies. By contrast, imaging techniques that acquire molecular information are only partly used in clinical and preclinical practice. The relevant imaging technologies have their own characteristics, but none of them is flawless. They have limitations to varying degrees in sensitivity, spatial resolution, imaging depth, imaging objects, etc.[14-19]. The use of these imaging techniques is complementary when conducting scientific research. Researchers can use their respective advantages to achieve their own research purposes. Developing new imaging technology or improving the existing imaging technology, and expanding the application field is also the concern of researchers.

In these Bioimaging techniques, luminescence imaging is perhaps the fastest-growing technique. The luminescent phenomenon was known in the mid-19th century. In the early 20th century, luminescent imaging technology was applied to the medical field. In recent years, luminescent imaging has developed rapidly, such as tomography reconstruction technology based on near-infrared light, spectral separation technology, image fusion technology, and multimode imaging. Some imaging systems used in preclinical and clinical have been commercialized [20,21].

The key to luminescence imaging technology is a luminescence probe.

2. Luminescent probe

2.1. mechanism and optical parameters of the luminescent probe

The luminescent probe is an important contrast media in luminescent imaging technology. To select, design, and develop an appropriate luminescent probe, it is necessary to deeply understand the mechanism of the luminescent probe. In short, when the excited electrons transition, the luminescent substance moves from the ground state of a single state (S\(_0\)) to the one-line excited state (S\(_1\)) with high energy. In the process of de-excitation, the excited electrons dissipate energy and return to the ground state (S\(_0\)) by internal conversion or return to the ground state (S\(_1\)-S\(_0\)) by radiation energy, named fluorescence emission (Fig.1 a); Regarding phosphorescence emission (Fig.1 b), the electrons in the S\(_1\) state can pass through the system to reach the triplet excited state (T\(_1\)) and then return to the ground state by radiating energy; In some special cases, T\(_1\) electrons state can reversibly return to S\(_1\) and then return to the ground state by means of radiation energy, which is called delayed fluorescence (Fig.1 c) [22-28].

![Schematic diagram of typical fluorescence, phosphorescence, and delayed fluorescence luminescence mechanisms](image)

Fig 1. Schematic diagram of typical fluorescence, phosphorescence, and delayed fluorescence luminescence mechanisms [27].

At present, several parameters involved in the luminescence process are used for both detecting and imaging (Fig.2). The luminescent quantum yield can be obtained by measuring the luminous intensity \(I\) at the given excitation and emission wavelengths (usually the maximum excitation and emission wavelengths). However, it is often cumbersome to evaluate luminescence by measuring the quantum yield of luminescence. In general, the measurement of luminous intensity \(I\) is used to replace the
measurement of luminous quantum yield to evaluate the luminous situation, and $F$ is usually expressed in any unit, which is only compared and analyzed under the same instrument and environmental conditions.

Anisotropic $R$, as a function of the luminous intensity acquired at both vertical and horizontal orthogonal polarization angles, reflects the molecular rotation rate during the luminescence lifetime ($\tau_F$). Under ideal conditions, it is an inherent property of luminescence and is not affected by test tools.

Luminescent lifetime $\tau_F$, which is a function inversely proportional to the decay rate of the excited state radiation. Through measuring them, we can extend the analysis of these quenching effects to the time domain and determine the rates of reaction regard various excited states. The lifetime can be obtained by fitting the attenuation curve directly measured by pulse or periodic modulation excitation. Because these radiation attenuations reflect the intrinsic properties of excited molecules in a specific environment, the test is not affected by the instrument.

The emission spectrum refers to the corresponding relationships between emission intensity $F$ and emission wavelength $F(\lambda_{em})$, where $F(\lambda_{ex})$ is obtained when intensity is monitored at the excitation wavelength, reflecting energies involved in transitions between different ground states. In addition, the weak noncovalent intermolecular interactions can be closely monitored by $F(\lambda_{em})$, and the excited state of the reaction can result in significant change of $F(\lambda_{em})$.

![Fig 2. Several optical parameters and detection methods used for sensing and imaging [29].](image)

The optical parameters for luminescence measurement are very limited. Therefore, the combination of a single test can enrich the detection and evaluation methods and even obtain some useful information related to the luminescence characteristics of the system. However, the instruments and analyses based on combinatorial testing are often complex. These techniques are only suitable for a few specific experiments and can not be popularized. Therefore, it is necessary to find some research methods that provide rich information and operate simply, reliably, and practically.

Ratio detection based on luminous intensity (recording intensity ratio of two or more wavelengths) provides this possibility. It has the characteristics of rich content, simple and convenient method, and strong reliability. It can be used in a conventional microscope, flow cytometry, and plate reader. In addition, these methods can provide extremely high sensitivity and anti-interference even at low absolute intensity levels. [30,31] One of the key factors for ratio detection and imaging is to have a new ratio probe suitable for this detection method. Next, we will briefly introduce the state-of-the-art ratio luminescence probes and their applications.

2.2. Research progress of luminescent ratio probe

The biggest problem of the optical imaging-based traditional luminescent probe is that the inaccurate test results would occur because something has nothing to do with the analytes, which might result in the absolute signal fluctuation collected, in the case of imaging relying on the absolute luminous intensity to generate signal output. On the contrary, ratio detection and imaging can realise more sensitive and more reliable test results by providing automatic calibration. Generally, there are two types
of design strategies of luminescence probe being appropriate for the ratio detection and imaging: the first is that people could bring into the second light-emitting unit being insensitive to the analytes as the reference signal based on responsive luminescence. The second strategy is that people can increase the emission ratio variation by using the two different light signals, generating different opposite tendencies when facing different analytes. In this part, we will introduce some small molecule luminescent probes and nano/Polymer luminescent probes.

2.2.1. Introduction of the ratio-type small molecule luminescent probe. As we all know, the design and preparation of the small molecule luminescent probe are significantly important to the development of light imaging. The small molecule probe has a stable construction, good repeatability, good concise of biosafety research, etc. However, in the traditional optical detection and imaging, there are some problems of quantitative analysis of analytes by only using a single emission zone provided because all kinds of factors being independent of analytes. Thus, to avoid the false negative or false positive imaging results, people must bring into another emission belt being used to construct a ratio type small molecule probe based on the original emission belt to realize the ratio signal with the self-calibration property. In this section, I will briefly introduce the construction strategy and application of the luminescent ratio probe.

Chromophores with characteristics of ESIPT, always refer from hydroxy (or amino) part to ketonic oxygen (or the amino nitrogen) atoms fast proton transfer, based on and presents two olefinic alcohol and ketone in the form of. Under many conditions, the variations of the relative strength of two emission bands (respectively from the form of enol and ketone) based on the ESIPT chromophores can be used as the foundation of ratio sensing and imaging. Moreover, other dyes with ESIPT properties, including 2- (20-hydroxy phenyl) benzothiazole (HBT) and 3-hydroxy flavone (3HF), have been used to construct ratio type probes. For example, Demchenko et al. reported that dyes based on 3HF could be used as a luminescent membrane probe to monitor the cell's early apoptosis through ratio type detection and imaging techniques [32]. Also, as N-3-BTTPB for the colloidal solution of fluorine ion detection has been proved to be effective (Figure 3 b, c), it has the ppb level of sensitivity [33]. However, based on the ratio of ESIPT detection has been proved to be limited in some cases because the probe in the water of protons may cause interference detection results.

Fig 3. (a)Mechanism of using BTTPB as chemosensor for the detection of NaF; absorption (b) fluorescence (c) spectra of BTTPB [33].
Generally, FRET and TBET always include energy transfer of chromophore between donor and receptor. Hamachi. et al. reported a zinc ion luminescence probe based on FRET with two Zn$^{2+}$ binding sites [34]. In the probe, cumarin is an energy donor, and xanthene is an energy receptor (Figure 4). This dinuclear complex combining of Zn$^{2+}$ can be used as a probe for detecting the triphosadenine ratio in the living cells in a neutral HEPES buffer.

**Fig 4.** Dual-emission detection of ATP using chemosensors (a); ratiometric contour of 4-2Zn (II) stained living cells (b,c,d) [34].

For TBET, as part of the energy to the body and as part of the energy, receptors are linked together by a conjugate. Tang et al. reported a TBET based such as luminescent probes, can be used for the ratio of mercury ion in the cell type detection and imagine, as shown in Fig. 5 [35]. In the probe, by using the four phenyl vinyl as the energy donor, and rhodamine B as the energy receptor, the energy transfer efficiency reached 99.9%, and Pseudo Stokes shift to 280 nm. After the reaction with mercury ion, the long wave area of emission band significantly enhanced with a short wave period of emission abating, realizing the ratio value ($I_{595nm}/I_{480nm}$) about 6100 times growth.

**Fig 5.** Sensing mechanism of the ratiometric Hg$^{2+}$ for detecting p/m-TPE–RNS (a); PL spectra of altered amounts of Hg$^{2+}$(b); microscopic images of HeLa cells (c) [35].
The cream base association is an exciting states collision complex formed by charge transfer interaction between the excited state of the light-emitting unit to determine the amount of chemical and with the same or different kind of ground state of the colored light group [36,37]. Cream base of association luminescence emission characteristics are different from monomer unit (not associating the light-emitting unit). The launch of the emission spectrum usually has a wavelength redshift and no vibration of the structure of the emission spectrum feature (resulting in a wide launch). Based on the analysis of the differences, content-driven cream base associating the separation of content and the change of relative orientation can lead to monomer and cream base association luminescence ratio value changes. Pyrene is considered one of the most commonly used sensor molecules for analysis detection based on the Cream Base Association. It has monomer emission from 370 to 380 nm and cream base association emission from 460 to 480 nm.

Schmuck et al. reported polypeptide beacon based on pyrene. It can effectively interact with double-stranded DNA (Figure 6) [38]. The folded form of polypeptide beacon presents a typical pyrene cream base association emission peak in the initial state. Based on one's experience of polypeptide beacon from folding form to stretch form of conformational change after combining double-stranded DNA. In the change process, the peptide beacon light-emitting change from emission dominated by cream base association (490 nm) to emission dominated by monomers (406 nm). Therefore, monitoring the two kinds of the relative intensity of emission peak ($I_{406}/I_{490}$) can be used to detect double-stranded DNA.

2.2.2. The Introduction of Ratio Type Nano/Polymer Luminescent Probe. Compared with the traditional probe based on the small molecule, nanoprobes, because of the inherent optical and physicochemical performances, can effectively improve the optical sensing and imaging of the sensitivity, specificity, targeted, and potential of constructing a multimode detection and imaging [39]. Using a nanometer probe as a substitute for a small molecule probe can significantly improve detection and imaging technology performance, but relying on the absolute strength based on a single launch nano-optical probe of signal collection a lot of the time is not satisfactory. It still is under the influence of probe concentration uncertainty, laser power instability, and so on. Below, we will briefly introduce the ratio of nanometer probe-type optical design and the application of the latest progress for five categories (Figure 7): the double dyes embedded nanoparticles, attached on the surface of the nanoparticles with dye nano compound, hybrid nanoparticles, inherent dual emission of nanoparticles, nanoprobe based on the structure of DNA.

![Microstructure and binding mode](image-url) [38]

**Fig 6.** Microstructure of 1 (a); Binding Mode of 1 Bound top (dA·dT)2 (b); the image displaying the corresponding cuvettes under UV light (c) [38].
Dual dyes embedded in nanoparticles

Usually, at least two fluorescent dyes along with various emission bands are combined with the nanoparticles to prepare dual-emission nanoprobes for ratio type detection. One of the luminescence units is used as a reference, and the other is used as a response luminescence unit to achieve the ratio signal output. A nanoluminescent probe, called PEBBLE (probes encapsulated by biologically charged embedding), is prepared and applied to determine intracellular pH by ratio method. Inspired by this work, researchers have replaced the above nanomatrix with silica, liposomes, polymers, nanogels, and metallic organic framework (MOF) [12,40-47] and designed and fabricated various Pebble ratio nanoparticle probes. Sung and co-workers successfully fabricated a FRET-based nanoprobe to detect and imaging intracellular pH [48]. The nanoprobe was prepared by binding pH-sensitive conformational N-palmitoyl chitosan (NPCs) to the donor portion (Cy3) and the acceptor portion (Cy5) of FRET (Figure 8). Spectral studies have shown an enhanced in the Cy5/Cy3 ratio. The ratio luminescence imaging shows a significant color change, indicating that the as-synthesized probe can efficiently locate pH changes. The figure shows that the nanoprobes designed are well suited for proportional tracking of pH changes in living cell environments.

Fig 7. Several design strategies for ratio type nano-luminescent probe [39].
Fig 8. FRET nanoprobes for pH-responsive detection and their working principle (a); mapping spatial pH changes in living cells(b); FRET measurements (c,d,e) [48].

The dual-emission nanoluminescent probe above overcomes many problems encountered by traditional single-emission nanoluminescent probes and significantly improves the sensitivity and dependability of detection and imagination technology. Nevertheless, this sensing system typically requires at least two fluorescent molecules, which raises manufacturing and cost issues.

Nanomaterials with dyes attached to the surface of nanoparticles
Nanoparticle-dye type nanocomposites for ratio type optical detection and imaging are typically prepared by using fluorescent dyes with different emission properties attached to the surface of nonluminescent nanoparticles that act as carriers, wherein one fluorescent dye is insensitive to the target analyte. It can be used as a reference for ratio pairing, while the other response specifically to the target analyte. Due to the advantages of excellent biocompatibility and cell penetration [49,50], gold nanoparticles (AuNP) have been widely used as non-luminescent nano-particle scaffolds for the realization of double-emission nano-dye complexes.
Yu et al. proposed a luminescent nano-probe for luminescent imaging of pH ratio in cells and in vivo [51]. The "gold nanosubmarine" consisted of AuNPs as nanocarrier, and pH-sensitive thiol-rhodamine and luciferin derivatives were modified onto AuNPs via the Au-S bond, respectively (Figure 9). With increased pH, the luminescence from 510 nm of luciferin increased significantly, while the luminescence from 580 nm decreased gradually. The ratio luminescence imaging shows that the ratio values based on the two luminescence intensities show a decent linear correlation from pH 6.0 to 8.0. After successfully application in living cells, they used Balb/c-nu mice as research subjects to perform pH sensing and imaging experiments in vivo.

DNA-based nanoparticle structure.

Due to the exceptional specificity and adaptability of molecular identification, nucleic acids became suitable for constructing various functional nanoscale luminescent probes. Krishnan et al. reported a Clensor for chloride detection [52]. As shown in Figure 10 a, a Clensor is built based on three parts: sensing (P), reference (D2), and targeting (D1). The sensing part P is the nucleic acid sequence of 12 polymers labeled with dye (BAC) and sensitive to chloride. The reference part D2 was the DNA sequence of the chloride-insensitive 38 polymers labeled with a fluorescent dye (A647). The targeted portion of D1 is the DNA sequence of poly 26. Subsequently, D1 was tuned with a completely characterized Tfapt to form a D1Tfapt (Figure 10 b). The spectral study shows that chloride can induce the luminescence quenching of BCA at 505 nm, while the luminescence of A647 at 670 nm remains unchanged. In the concentration range of 5-200 mM, the luminescence ratio value I_{670 nm}/I_{505 nm} exhibits a close linear relation with chloride. In addition, the design of Clensor is also used to detect the ratio of chlorine ions in living intracellular body detection.
2.3. Research progress of time-resolved fluorescence probe

2.3.1. Time-resolved optical detection and imaging principle. In the process of detection and imaging, the instability of laser power and the uncertainty of probe concentration in the organism will reduce the imaging quality and the accuracy of quantitative analysis. The ratio detection and imaging method can effectively solve the above problems. However, it is still difficult to accurately identify the luminescence signal from the probe. Time-resolved fluorescence technology provides a new idea, that is, according to different fluorescence decay rates, fluorescence from the probe and background fluorescence (autofluorescence) can be distinguished in time dimension [27]. Next, the detection and imaging principles of two time-resolved luminescence imaging technologies are briefly introduced.

Time-gate luminescence imaging technology (TGLI)

The traditional luminescence analysis and microscopic imaging technology are based on the steady emission intensity of a specific wavelength to observe and analyze the composition and location of a specific analyte. However, when the analyte concentration is relatively low, or the probability of occurrence is very small, identifying luminescence signal from the substances with autofluorescence will be hard. Time-gated luminescence (TGL) technology can be used to overcome this challenge. TGL measurements are typically conducted with a pulsed light source to excite the target probe. Until the short-lived signal decays beyond the detection limit, the detector remains closed to ensure that only the long-lived signal will enter the collection window (Fig. 11). Even if the emission intensity is very weak, these long-lived target signals can be distinguished from short-lived interference signals in the dimension of time, which greatly improves the sensitivity and accuracy of detection. Regarding the signal-to-noise ratio, when the short-lived interference signal gradually decays, it gradually increases. When the attenuation is complete, it reaches the maximum. After that, the value gradually decreases as the number of long-lived signals that can be collected becomes less.
Fluorescence lifetime imaging (FLIM)

The FLIM method measures the luminescent lifetime of probes in cells, tissues, and small animals for the purpose of determining their spatial distribution. By analyzing the emission lifetime, the long-life signal is easily separated from the short-life background and scattered light signals. In addition, it can be used to obtain relevant information by monitoring the change of the lifetime, such as the change of the microenvironment of the probe or the change after the interaction with some substances in the probe and the surrounding environment. Using the probes whose emission lifetime depends on the environmental factors, the imaging images of ion concentration, oxygen content, pH value, and temperature can be obtained. In addition, the interaction of electrons between the probe and its surrounding environment FLIM image.

FDPM and TCSPC methods are generally used in life measurement in PLIM. In frequency domain measurement, compared with the excitation, the luminescence of the sample shows a lower modulation and a lagging phase (Δφ). The emission decay curve is reconstructed by the phase difference between them. The emission lifetime is obtained (Fig. 12 a). TCSPC is another mature technology for luminescence lifetime measurement. It measures each photon's time arriving at the detector after the probe is excited by the pulsed laser (Fig. 12 b). After the sample is repeatedly excited by a high-frequency light source, photon probabilities are used to fit the emission decay exponential function.
related to the lifetime to obtain the parameters related to the emission lifetime. Fluorescence lifetime imaging in the picosecond and nanosecond range is rapid and effective, while imaging luminescence lifetime in microseconds or milliseconds requires a longer time as low repetition rate stimulated pulse excitation is always required.

Both TGLI and PLIM can provide decent imaging with minimum autofluorescence interference. TGLI achieves higher signal-to-noise ratio imaging by collecting long-lived fluorescence and eliminating short-lived fluorescence for analysis, which will lose part of the luminous intensity. The intensity of TGL is linear, with the concentration of the probe in a certain range. Being independent of probe concentration, the lifetime can be used as a measure to construct images. The probe with a super long life is more suitable for TGLI technology.

2.3.2. Introduction of organic time-resolved optical detection and imaging probe. Luminescent imaging is receiving extensive attention because of its excellent sensitivity and specificity. Imaging-based on luminescent compounds can usually show the location of luminescent compounds in cells. Nevertheless, due to the spontaneous fluorescence of biological samples, signals will also be generated in the process of cell imaging. It is difficult to distinguish luminescent compounds and biological autofluorescence in imaging results, which will greatly reduce the sensitivity and reliability of imaging. Fortunately, unlike the long-lived luminescence probe, the spontaneous biological fluorescence decay rate is very fast, which is easy to distinguish in the time domain, and the interference caused by the spontaneous biological fluorescence can be easily removed by time-resolved luminescence imaging technology. In this paper, we introduce four long-life luminescent probes for time-resolved detection and imaging: (1) organic compounds [53], (2) Inorganic nanomaterials [54-56], (3) Lanthanide [57-59], (4) Phosphorescent transition metal complexes [60-65].

TRL detection and imaging based on the lanthanide.

Due to the reversible system crossing interaction between singlet and triplet, organic compounds with delayed fluorescence emission tend to have a relatively long emission lifetime. However, it is easily quenched by oxygen. Song reported an organic compound dcf-mpym which can be used for biological detection and imaging. By two α β- Unsaturated carbonyl modified compounds can be used as long-lived fluorescent probes (DCF mpym thiol) [66]. Dcf-mpym-thio, quenched by unsaturated carbonyl but does not emit light, reacts with Cys to form dcf-mpym.

Yuan also reported a kind of europium (III)/ terbium (III) complex H2S probe similar to the pet mechanism [67]. There is a 2,4-dinitrophenyl part in it, which has a pet effect. When it reacted with H2S, the dinitrophenyl was broken, the pet effect was eliminated, and the probe luminescence was enhanced. However, the luminescence of the green TB (III) compound increased significantly, the red emission of the EU (III) complex decreased slightly due to intramolecular charge transfer. Combined with TGL technology, two emission intensity ratios (green/red) is applied for quantitatively detect H2S in solution and cells. As shown in Fig. 1.3, after NaHS was added, the cells showed strong green light without spontaneous fluorescence through time-gated luminescence imaging.

![Image](image_url)

**Fig 13.** The application of europium (III)/terbium (III) complex binding time-gate luminescence imaging technique for intracellular H2S detection reported by Yuan's group [67]
Yuan reported weak luminescent europium (III) complex with o-diaminophenyl moiety (Fig. 14) [68]. Due to the PET effect of the o-diaminophenyl part of the probe, the initial luminescence of the probe is very weak (almost no luminescence). When reacting with no, o-diaminophenyl will be converted into electron-deficient benzotriazole derivatives, which will promote the recovery of luminescence. The EU (III) complex luminescence is enhanced 47 times by adding no to the solution, and the lifetime show an increase from 1.08 ms to 1.30 ms. Combined with TGLI technology, the complex can be used to detect no in onion endothelium. By incubating the cells with the probe, a strong luminescence signal shown in the cells; After pretreated with no scavenger (c-ptoio), no signal was detected in the cell. These results indicate that without c-ptoio, the observed luminescence is due to the reaction of the EU (III) complex with NO in cells to produce EU (III) complex containing benzotriazole. In addition, in the quantitative analysis experiment, the europium (III) complex probe is used to measure that the concentration of NO released from the onion inner epidermis (50 mg) in the cell culture medium for 60 minutes is about 50 nm.

Fig 14. Eu (III) complex probe binding time gate luminescence imaging technique reported by Yuan's group for the detection of intracellular nitric oxide [68]

TRL detection and imaging involving transition metal compounds

Compared with lanthanides, the phosphorescent transition metal complexes have more triplet excited states [69-73]. These complexes, based on the types of central coordination metal and ring metal ligands, can form a variety of excited states. There is a spin barrier in the transition metal complexes, and the luminescence lifetime of triplet states is relatively long. The properties of these excited states are easily affected by the changes in microstructure and the external environment. Based on these characteristics, combined with TRLT, long-life phosphorescent transition metal complexes can be used to design and prepare various biological probes suitable for a complex environment.

Zhao reported an amino acid probe containing two amino acids α, β- Phosphorescent iridium (III) complex with unsaturated ketone group [74]. Based on its long luminescence lifetime, even with a high concentration of fluorescent rhodamine B, the titration detection of Cys can be realized by TGL technology with a delay time of 100 ns. The probe could be excited by two photons (800 nm). Combined with two-photon imaging, tgl, and PLIM technology, the probe can be used to detect Cys in cells (Fig. 15). Zhao also reported that the complex probe was introduced into mesoporous silica spheres with blue light emission by the physical adsorption method. A ratio probe for Cys detection and imaging was prepared.
Fig 15. A phosphorescent iridium (III) complex probe combined with two-photon, lifetime imaging was reported by Zhao's group for intracellular Cys detection [74]

PTMC has trilinear phosphorescence emission, which is easily affected by O$_2$. Zhao reported a cationic conjugated polyelectrolyte luminescent probe based on a blue fluorescent polyfluorene (O$_2$ insensitive) and a red phosphorescent porphyrin platinum (II) complex (O$_2$ sensitive) [75]. In this probe, the fluorene unit can be used as the donor of the fret, and the porphyrin platinum (II) complex can be used as the acceptor. When the ambient O$_2$ content decreases, the luminescence from the porphyrin platinum (II) complex is enhanced, while that from polyfluorene is almost unchanged (Fig. 16). Through PLIM, TGLI, and ratio luminescence imaging, the probe can monitor intracellular O$_2$ and image hypoxia in nude mice tumors.

Fig 16. Porphyrin Platinum (II) complex - polyfluorene probe binding lifetime image reported by Zhao's group for oxygen detection in tumor cells [75]

Zhao designed and prepared a kind of gold nanoclusters (AUNCS) grafted with phosphorescent Ir (III) complex for hypoxia detection in zebrafish [76]. The emission peaks of the probe are at 510 nm and 590 nm, respectively, and only the 590 nm emission peak from the phosphorescent iridium (III) complex is obviously dependent on the oxygen concentration (Fig. 17). When the probe was injected into zebrafish, two bandpasses (520±20 nm, 600±20 nm) were observed, and the luminescence lifetimes were 133 ns and 127 ns, respectively. When zebrafish were treated with 2,3-butanedione
MONOOXIME (BDM), inducing brain hypoxia by eliminating myocardial contractility, it was observed that the lifetime of phosphorescence from zebrafish brain collected by band-pass(600±20nm) was extended to 331 ns.

Fig 17. Iridium (III) complex and gold nanocluster probe binding lifetime imaging reported by Zhao's group for the detection of cerebral hypoxia in zebrafish [76]

Nangaku realized the quantitative detection of oxygen concentration in mice based on the life span of IR emitting iridium (III) complex [77]. They measured the phosphorescence lifetime of iridium (III) complex under different O_2 partial pressures in HK-2 cell culture medium and constructed the calibration curve of phosphorescence lifetime with oxygen concentration (Fig. 18). On this basis, they studied the phosphorescence lifetime of iridium (III) complex in mouse kidneys under three hypoxia conditions: acute ischemia, hypoxemia, and anemia. For example, when the iridium (III) complex was mainly distributed in renal tubular cells 30 minutes after intravenous injection, the phosphorescence lifetime of the iridium (III) complex was immediately prolonged by clamping the renal artery to induce acute renal ischemia; Once the clip is removed, the phosphorescence lifetime is restored. When mice inhaled 15% O_2, the life of phosphorescence was prolonged reversibly.

Fig 18. The phosphorescence iridium (III) complex binding life test reported by the Nangaku group for the quantitative determination of oxygen in mice [77]
3. Conclusion

In this review, we first briefly outlined bioimaging techniques and their development. Then we introduced the mechanism of luminescence probe, which is the key to realize luminescence imaging. We explained the imaging principles of ratio-based luminescence probes and time-resolved luminescence probes and introduced their research progress.

There are some problems, such as shallow tissue penetration depth and interference of biological tissue autofluorescence. Luminescent imaging has been widely used in life science, medicine, drug development, and other fields due to its remarkable sensitivity, superior spatial resolution, small damage, as well as real-time monitoring. With the development of luminescent imaging, researchers can deal with and even solve some major problems in tumor molecular science now. For example, how the components of intracellular signaling pathways interact and influence each other; what is the dynamics and flux rate of this association between them; what is the difference between this association in Malignant and normal cells; Can humans use these variances to produce less toxic and more effective drugs. Therefore, it is of positive significance to promoting the development of research work in the field of luminescence imaging. The following is a brief introduction of the luminescent imaging system and the luminescent probe for this imaging technology.

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