

Chapter 24

Development of Novel Fluorogenic Probes for Realizing Rapid Intraoperative Multi-color Imaging of Tiny Tumors



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24.1 Rational Design of Organic Fluorogenic Probes Based on Unique Spirocyclization of Rhodamines by the Intramolecular Hydroxymethyl Group

Fluorescence imaging is one of the most powerful techniques currently available for continuous observation of dynamic intracellular processes in living cells. In recent years, this technique is not restricted to biological experiments but applicable to clinical fields. Among them, intraoperative fluorescence imaging of lesions or structures that are difficult to identify with the naked eye enable real-time characterization of tumors, thereby aiding clinical decisions on both intra- and postoperative treatments (Stewart and Wild 2014; Hanahan and Weinberg 2000).

Suitable fluorogenic (activatable fluorescence) probes that are initially non-fluorescent and turn to be highly fluorescent upon binding/reaction with target biological molecules of interest, are naturally of critical importance for fluorescence imaging. We have been working for developing novel fluorogenic probes, and succeeded to construct several versatile rational design strategies based on the concept of photoinduced electron transfer (Miura et al. 2003; Urano et al. 2005) and intramolecular spirocyclization (Kamiya et al. 2011; Sakabe et al. 2013). As for the latter design strategy, we have synthesized and evaluated a series of hydroxymethyl rhodamine derivatives and found an intriguing difference of intramolecular spirocyclization behavior: the acetylated derivative of hydroxymethyl rhodamine green

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(Ac-HMRG) exists as a closed spirocyclic structure in aqueous solution at physiological pH, whereas HMRG itself takes an open non-spirocyclic structure. More precisely, the pK_{cycl} value, the pH at which the absorbance or fluorescence of the compound decreases to a half of the maximum value as a result of spirocyclization, of HMRG was calculated to be 8.1, but that of AcHMRG where one of the amines on the xanthene moiety was amidated, decreased to 5.3. Therefore at the physiological pH of 7.4, HMRG predominantly takes the ring-open form with strong fluorescence at λ_{fl} of 524 nm, but its acetylated derivative AcHMRG would predominantly be in the spirocyclized closed form with reduced absorbance or fluorescence in the visible region.

24.2 Development of Novel Fluorogenic Green Probes for Biological and Medical Purposes, Especially for Intraoperative Rapid Tumor Imaging

Based on the above-mentioned findings, we have established a general design strategy to develop highly sensitive fluorogenic probes for proteases and glycosidases: Converting the acetyl group to different functional groups or peptides that are targeted by proteases and glycosidases would enable a wide range of probes to be developed. Specific cleavage of the substrate moiety in the non-fluorescent probe by the target enzyme generates a strong fluorescence signal. In order to confirm the validity and flexibility of our strategy, we designed and synthesized fluorescence probes for leucine aminopeptidase, fibroblast activation protein, cathepsin B/L, β -galactosidase, and so on (Fig. 24.1a). All these probes were almost non-fluorescent due to the formation of spirocyclic structure, but were converted efficiently to highly fluorescent HMRG by the target enzymes (Fig. 24.1b). We confirmed that the probes can be used in living cells. These probes offer great practical advantages, including high sensitivity and rapid response (owing to regulation of fluorescence at a single reactive site), as well as resistance to photobleaching.

For medical imaging purposes, protease including aminopeptidase activities are known to be important imaging targets, because they play essential roles in many diseases, and some of them show altered expression levels in the pathological context (Hanahan and Weinberg 2011; Weigelt 2005). For endopeptidase, there already developed many fluorogenic or FRET-based probes such as those for MMP and cathepsins, but there were few examples for aminopeptidases, especially those function with visible light irradiation. Therefore, by making use of the above-mentioned strategy based on the spirocyclization, we started to develop many fluorogenic probes for aminopeptidases. For example, gGlu-HMRG, a novel spirocyclized rhodamine-based fluorescence probe for γ -glutamyltranspeptidase (GGT), which is well-known to be upregulated in various cancer cells (Pompella et al. 2006), was successfully developed (Fig. 24.2a). By applying gGlu-HMRG to various cancerous cell lines whose GGT activity is upregulated, fast enzymatic reaction of gGlu-HMRG with GGT occurs on the plasma membrane to yield highly fluorescent

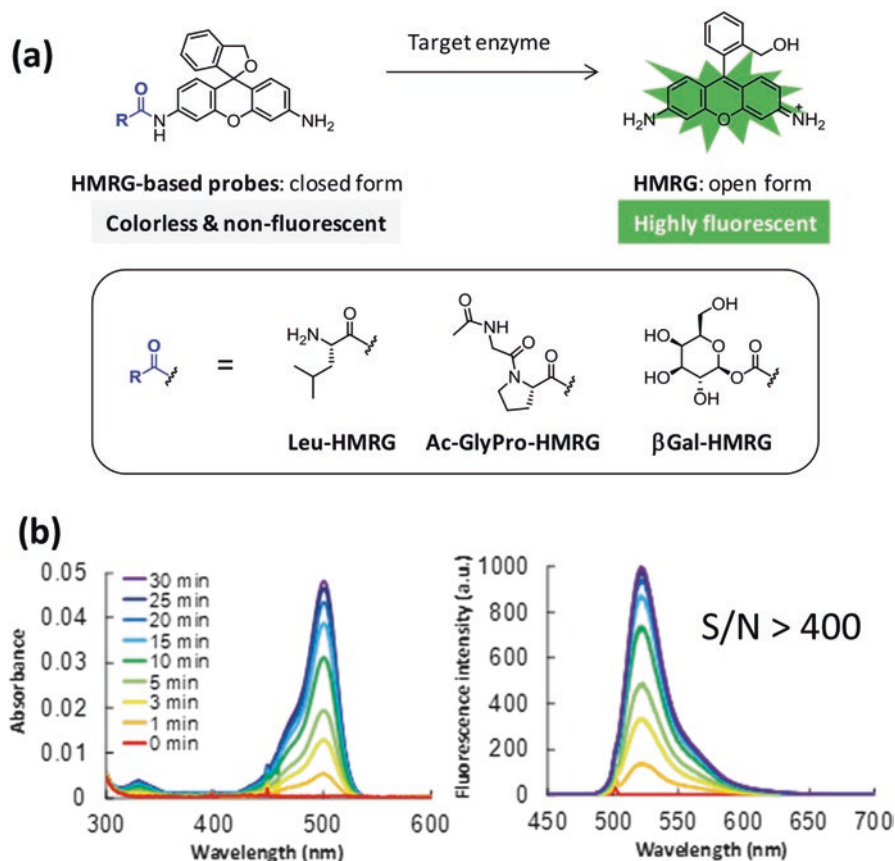


Fig. 24.1 Novel design strategy for various hydrolases-sensitive fluorogenic probes based on HMRG scaffold. **(a)** Enzymatic reaction of HMRG-based probes with the target enzymes. Leu-HMRG for leucine aminopeptidase (LAP), Ac-GlyPo-HMRG for fibroblast activation protein (FAP), βGal-HMRG for β-galactosidase. **(b)** Absorption and emission spectra of 1 μM Leu-HMRG at 0, 1, 3, 5, 10, 15, 20, 25 and 30 min after addition of LAP (0.04 U). Reaction was performed in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C. Excitation wavelength was 501 nm

product HMRG, which led us to establish a novel and highly activatable strategy for sensitive and fast-responding fluorescence imaging of tiny tumors *in vivo*. In mouse models of disseminated human peritoneal ovarian cancer, activation of gGlu-HMRG occurred within 1 min of topically spraying onto tissue surfaces that are suspected of harboring tumors, creating high signal contrast between the tumor and the background (Urano et al. 2011) (Fig. 24.2b, c).

Furthermore, not only with tumor mice models, but with freshly resected real human tumor samples, we started to examine the efficacy of gGlu-HMRG as an intraoperative tumor detecting agent by collaborating with many surgeons. Indeed, gGlu-HMRG was proved to be effective for rapid intraoperative imaging of breast (Ueo et al. 2015; Shinden et al. 2016), oral (Shimane et al. 2016), and hepatic

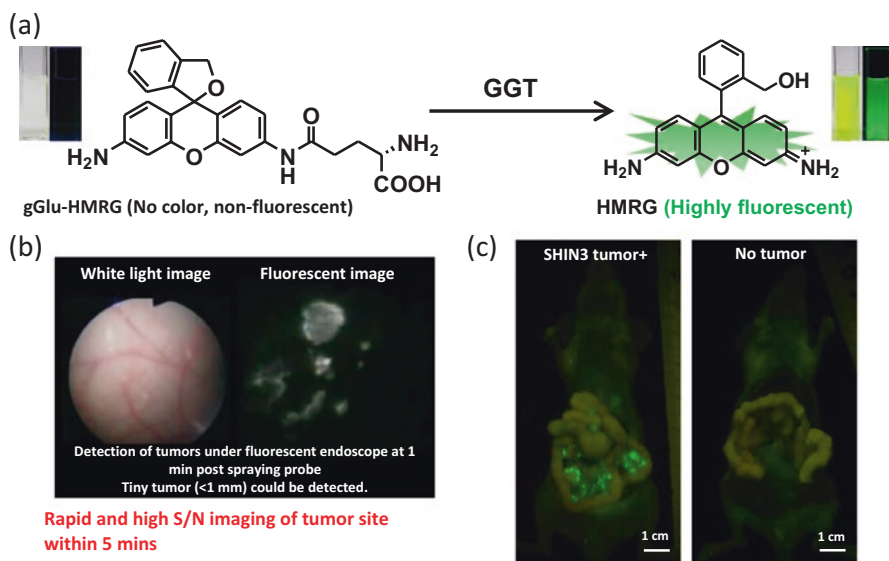


Fig. 24.2 (a) Novel HMRG-based fluorescence probe for γ -glutamyl transpeptidase. (b) Tiny disseminated tumor sites including those smaller than 1 mm in size could be clearly detected by topically spraying gGlu-HMRG after 1 min in the peritoneal cavity. (c) Tumor sites emitted very bright green fluorescence with high selectivity, which could be detected even with our naked eyes, only after 10 min post application of the probe

(Miyata et al. 2017) cancers. For example, breast cancer could be visualized regardless of its types like invasive/non-invasive or ER and HER2(+)/(−) with high specificity and sensitivity, both exceeded 90% (Ueo et al. 2015), and metastases in sentinel lymph nodes could be examined within a few minutes with high negative predictive values (>99%) (Shinden et al. 2016).

24.3 Development of Novel Fluorogenic Scaffold for Detecting Protease Activity in Longer Wavelength by Optimizing the Spirocyclization Properties: Novel Strategy for Fluorescence-Assisted Surgery with Multicolor Protease Imaging (Iwatate et al. 2016)

However, as can be easily imagined, because cancer cells are known to have big heterogeneity, gGlu-HMRG can only image cancer with elevated GGT activity, which accounts for only a small portion of all cancer. Two of the realistic clinical applications of fluorescent probes are preoperative diagnosis by fluorescent endoscopy and intraoperative diagnosis. Yet for fluorescence to be used for these purposes, the fluorescent probe must be able to detect, in principle, all types of cancer.

Although this is difficult to realize with a single probe targeting one protease, by spraying or administering a cocktail of fluorescent probes targeting various proteases that can cover for each other, it would become possible to simultaneously detect multiple proteases. If the cocktail is composed of probes with different fluorescence wavelengths, this would also enable the surgeons to distinguish and characterize the tumors according to the difference in protease activity profiles.

If protease probes that function in a similar manner to gGlu-HMRG but with different target and wavelength were developed, such imaging can be realized. For a protease probe to be used for multicolour imaging with HMRG, the scaffold dye must have a spectroscopic property that can be differentiated from HMRG. If the scaffold was a spirocyclizing rhodamine that bears an amine on xanthene moiety available for peptide conjugation to be the switch for fluorescence activation and retains the hydroxymethyl group on the benzene ring moiety of HMRG for intramolecular spirocyclization to occur, some of the favourable properties of HMRG may also be applicable for the new probes. Thus, modifying parts of HMRG was deemed to be an appropriate strategy to develop a series of protease probes for such purposes.

It would be more beneficial if the scaffolding rhodamine also met the following criteria:

- Has longer λ_{abs} and λ_{fl} than HMRG for better tissue penetration
- Emits fluorescence in the visible region for detection by the naked human eye
- Single reaction point for more sensitive and quantitative detection
- Photobleach-resistant

However, there were no reported rhodamines that met these criteria. Thus, the aim of the research was set to detect a variety of cancer with different protease activity profiles using small molecule based protease probes (Fig. 24.3).

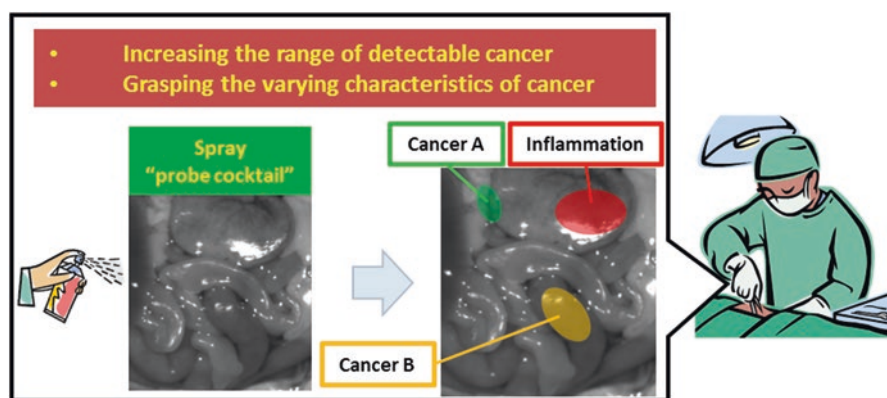


Fig. 24.3 A cartoon showing the outline of the Novel strategy for fluorescence-assisted surgery with multicolor protease imaging

For a rhodamine to function as a scaffold for a protease probe, it must have an unsubstituted amine for peptide conjugation. This meant that only one of the amines on HMRG could be modified, resulting in asymmetrical rhodamines. There were no reports on the synthesis of asymmetrical rhodamines bearing a hydroxymethyl group, therefore a synthetic route based on synthesis of symmetrical rhodamine was developed to prepare three asymmetrical rhodamine derivatives. The carboxyl groups of these asymmetrical rhodamines were then reduced to hydroxymethyl groups to afford three derivatives bearing a hydroxymethyl group: *N,N*-dimethyl derivative (HMDiMeR), *N,N*-diethyl derivative (HMDiEtR) and julolidine-fused derivative (HMJR) (Table 24.1: 1a, 2a, 3a).

Acetylated derivative of a rhodamine has been shown to function as a model compound for protease probes on HMRG. The changes to the absorption and fluorescence spectra matched well and most importantly, the change to pK_{cycl} can be modelled with an easy-to-synthesise compound with a single amidation reaction. Thus, acetylated derivatives of HMDiMeR, HMDiEtR and HMJR were also synthesised (Table 24.1: 1d, 2d, 3d).

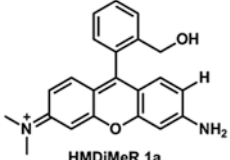
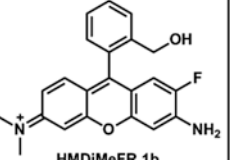
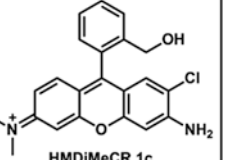
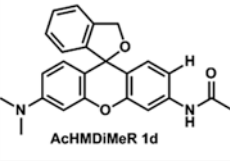
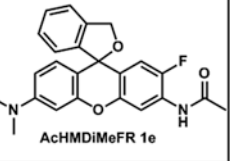
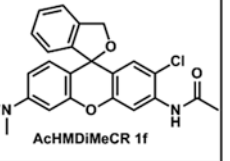
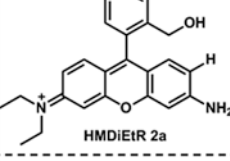
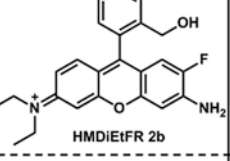
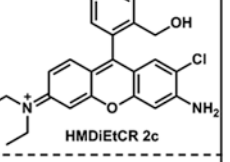
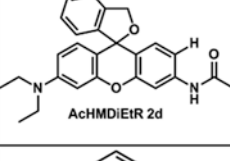
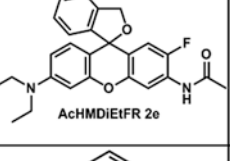
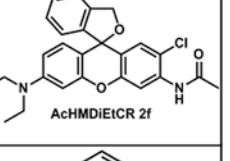
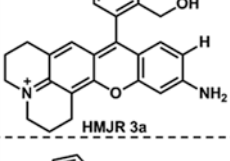
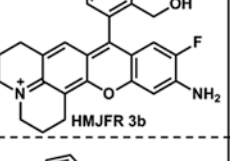
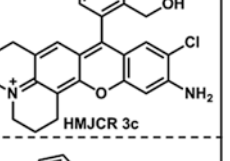
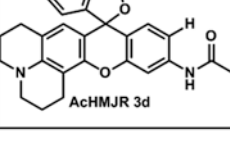
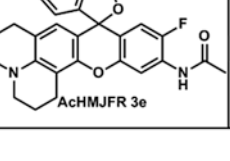
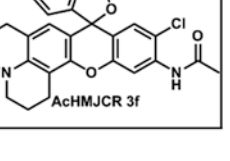
Examination of the spectroscopic properties of the synthesised compounds showed that λ_{abs} and λ_{fl} had indeed been extended (Table 24.2). As expected, julolidine-fused HMJR had the biggest red-shift with 46 nm for absorption. All of these rhodamine derivatives exhibited pH dependency by spirocyclization and their absorbance and fluorescence intensities decreased with increasing pH. Correspondingly, the pK_{cycl} values of the acetylated derivatives decreased to acidic values compared with their parental derivatives. The shift was around 2.7 for HMRG, HMDiMeR and HMDiEtR, but it was smaller for HMJR at 2.0. However, the values of pK_{cycl} generally increased to more basic values with the red-shifts. The pK_{cycl} values of acetylated derivatives were turned out to be ranging from 6.2 to 8.3, which were less suitable as scaffolds for protease probes, as protease probes based on them would not preferentially take the closed form at the physiological pH of 7.4. This would result in increased background fluorescence and smaller activation ratio.

As intramolecular spirocyclization occurs through intramolecular nucleophilic attack of the hydroxymethyl group on the 9 position of the xanthene moiety, a hypothesis that making the 9 position less electron-rich would reduce the pK_{cycl} value to the acidic side was contrived. One easy and synthetically-feasible solution was to introduce electron-withdrawing groups such as halogens to the xanthene moiety. Therefore, we started to synthesize three asymmetrical rhodamines with fluorination or chlorination to the 2 position of the xanthene moieties (Table 24.1: 1b, 1c, 2b, 2c, 3b, 3c), together with their acetylated derivatives. (Table 24.1: 1e, 1f, 2e, 2f, 3e, 3f).

The spectroscopic properties of the synthesised compounds supported the hypothesis that halogenation would have no negative effect on the spectroscopic properties such as λ_{abs} , λ_{fl} and Φ_{fl} , but would reduce pK_{cycl} values (Table 24.2). The reduction in pK_{cycl} values was larger for chlorinated compounds.

HMDiMeR derivatives and HMDiEtR derivatives were very dark compared to HMRG with Φ_{fl} of around 0.05, and their λ_{abs} were relatively short at around 530 nm: HMJR derivatives were brighter with Φ_{fl} of around 0.4 and had longer λ_{abs} at around

Table 24.1 Structures of synthesised asymmetrical rhodamines bearing a hydroxymethyl group and their acetylated derivatives

	X = H	F	Cl
HMDiMeR	 HMDiMeR 1a	 HMDiMeFR 1b	 HMDiMeCR 1c
AcHMDiMeR	 AcHMDiMeR 1d	 AcHMDiMeFR 1e	 AcHMDiMeCR 1f
HMDiEtR	 HMDiEtR 2a	 HMDiEtFR 2b	 HMDiEtCR 2c
AcHMDiEtR	 AcHMDiEtR 2d	 AcHMDiEtFR 2e	 AcHMDiEtCR 2f
HMJR	 HMJR 3a	 HMJFR 3b	 HMJCR 3c
AcHMJR	 AcHMJR 3d	 AcHMJFR 3e	 AcHMJCR 3f

550 nm. The pK_{cycl} value of AcHMJCR was 7.3, which suggests a half of the molecules would take open form under physiological pH, but this increases to 90% after the reaction. Even though these sets of values were not quite optimal in themselves, taking account the changes in Φ_{fl} and ϵ , fluorescence increase of about 40-fold could have been expected. Therefore HMJCR was chosen as the novel scaffold for a red-shifted protease probe.

Table 24.2 Spectroscopic properties of asymmetric *N*-alkyl rhodamines bearing a hydroxymethyl group

Compound	λ_{abs} (nm)	λ_{fl} (nm)	Φ_{fl}	$\text{p}K_{\text{cycl}}$
HMRG	501	524	0.81	8.1
AcHMRG	495	526	0.27	5.3
HMDiMeR 1a	529	553	0.05	8.9
HMDiMeFR 1b	532	559	0.06	8.2
HMDiMeCR 1c	536	559	0.04	7.7
AcHMDiMeR 1d	499	571	0.02	6.2
AcHMDiMeFR 1e	507	582	<0.01	5.7
AcHMDiMeCR 1f	505	581	0.01	5.2
HMDiEtR 2a	532	555	0.05	9.3
HMDiEtFR 2b	538	566	0.02	8.6
HMDiEtCR 2c	541	568	0.02	8.1
AcHMDiEtR 2d	501	573	0.01	6.7
AcHMDiEtFR 2e	508	583	0.01	6.1
AcHMDiEtCR 2f	507	578	0.01	5.5
HMJR 3a	547	569	0.50	10.3
HMJFR 3b	553	582	0.37	9.8
HMJCR 3c	555	582	0.42	9.1
AcHMJR 3d	513	589	0.11	8.3
AcHMJFR 3e	519	599	0.06	7.9
AcHMJCR 3f	517	592	0.07	7.3

The Φ_{fl} values were measured in 0.2 M sodium phosphate buffer at pH 7.4 for rhodamines or pH 2.0 for acetylated derivatives

As a proof of concept, HMJCR was used as the scaffold for a probe designed to target GGT, by replacing the acetyl group of AcHMJCR with a gamma-glutamyl group (Fig. 24.4).

The $\text{p}K_{\text{cycl}}$ value of gGlu-HMJCR was calculated to be 7.7, suggesting that approximately 33% of the probe exists in non-fluorescent spirocyclized form at the physiological pH of 7.4, whereas the putative hydrolysis product HMJCR with $\text{p}K_{\text{cycl}}$ of 8.9 exists predominantly in the fluorescent open form. In addition, the open form of gGlu-HMJCR was found to have remarkably reduced Φ_{fl} (0.06) and ϵ (20,000) compared to those of HMJCR (0.42 and 50,000, respectively). The combination of the diminutions in $\text{p}K_{\text{cycl}}$, Φ_{fl} and ϵ resulted in efficient quenching of the fluorescence of gGlu-HMJCR.

Then, gGlu-HMJCR was examined if it reacted with GGT *in vitro*. gGlu-HMJCR in aqueous solution was added commercially available purified GGT enzyme. A dramatic increase in fluorescence intensity of more than 30-fold was observed as a result (Fig. 24.5). gGlu-HMJCR was also proven to be stable in aqueous buffer at physiological pH. The product of the reaction between gGlu-HMJCR and GGT was analyzed by HPLC. The results confirmed that the putative hydrolysis product of reaction was indeed HMJCR. The above results suggested that gGlu-HMJCR could be recognised by GGT to be converted into HMJCR.

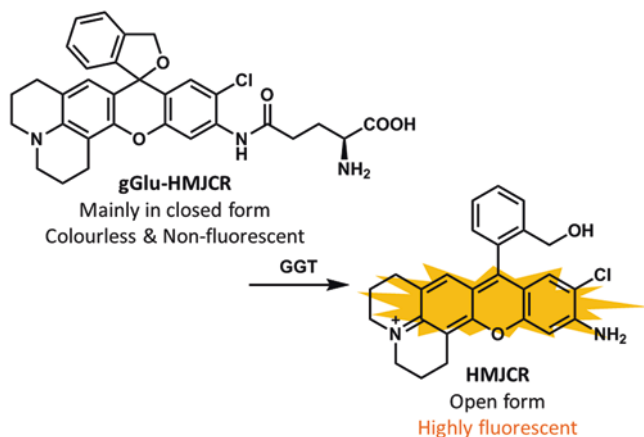


Fig. 24.4 Reaction scheme of gGlu-HMJCR

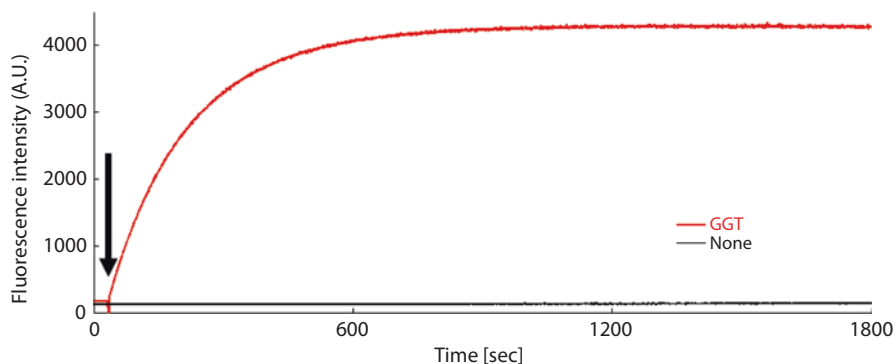


Fig. 24.5 Time course of enzymatic reaction of gGlu-HMJCR with GGT. Arrow indicates the time of GGT addition

Finally, an experiment was set up to discriminate two types of tumors by variation in protease activity profiles *in vivo*, using a cocktail of gGlu-HMJCR and previously reported Z-Phe-Arg-HMRG for cathepsins (Fig. 24.6) (Fujii et al. 2014).

Z-Phe-Arg-HMRG reacts with cysteine proteases in the cathepsins family such as cathepsin B and L, which are lysosomal endopeptidases with the increase in fluorescence of over 800-fold. As both cathepsins play a variety of roles in maintaining normal cellular metabolism, they are also naturally upregulated, secreted and show changes in localisation in various diseases, including breast, colorectal, gastric, lung, prostate, and ovarian cancers. These cathepsins are also known to contribute to the invasion of tumor cells to basal membrane, by degrading extracellular matrix components such as collagen I, collagen IV, laminin, fibronectin and elastin.

Mouse models of dual tumour implant were developed using two ovarian cancer cell lines with differing protease activities: SKOV-3/RFP and SHIN3. SHIN3 cells

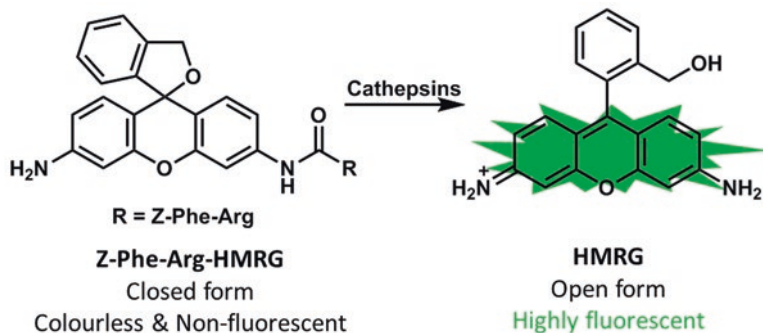


Fig. 24.6 Reaction scheme of Z-Phe-Arg-HMRG

exhibit both cathepsins and GGT activities, while SKOV-3/RFP cells only show cathepsins activity. Thus, whereas Z-Phe-Arg-HMRG should label both SHIN3 and SKOV-3/RFP derived tumors, gGlu-HMJCR should only label SHIN3 derived tumors.

Mice were injected intraperitoneally with 300 μl of PBS(-) containing 100 μM Z-Phe-Arg-HMRG and 1 mM gGlu-HMJCR, and sacrificed after 60 min incubation for fluorescence imaging with a Maestro. By unmixing the obtained images by HMJCR, HMRG, RFP and autofluorescence spectra, clear discrimination of two types of tumors with varying levels of protease activities by color *in vivo* was achieved (Figs. 24.7 and 24.8).

This was the first example known to date of such discrimination, and would serve as a guide for real-time and accurate characterization of tumor. The relationship between protease activity and therapeutic effect or prognosis are largely yet to be discovered, but such knowledge could be beneficial for aiding clinical decisions on intra- and postoperative treatments, leading to personalised medicine.

24.4 Conclusion

We have succeeded to construct a new fluorogenic scaffold HMJCR by optimizing its color, brightness, and spirocyclization property. HMJCR-based probes are capable of detecting various aminopeptidase activities in different color from so far-developed HMRG-based green probes. Thus, simultaneous live imaging of different protease activities in living cells, clinical specimen, and preferably in human patients in the future, are doable by the cocktail of HMRG- and HMJCR-probes.

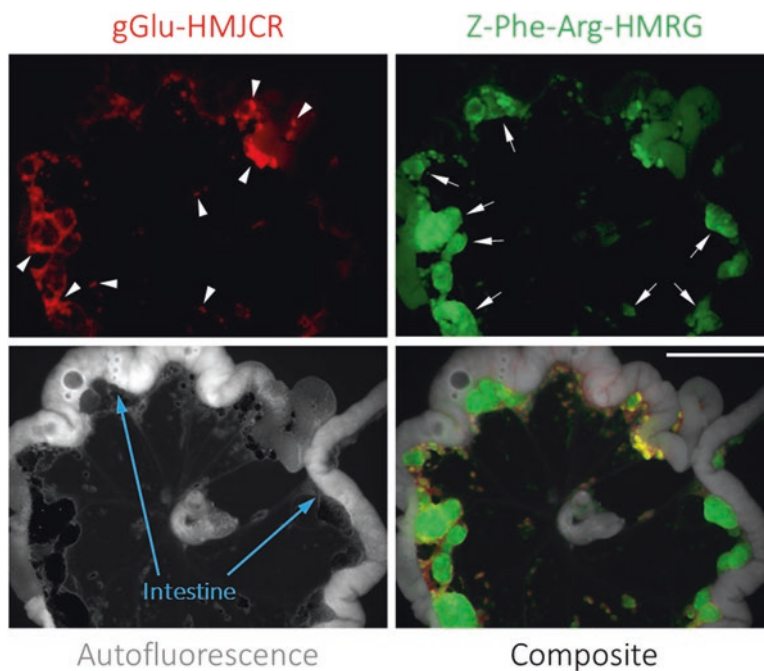


Fig. 24.7 Unmixed fluorescence images of a mouse model of dual peritoneal metastasis, at 60 min after combined intraperitoneal injection of Z-Phe-Arg-HMRG (100 μ M) and gGlu-HMJCR (1 mM). Arrowheads indicate SHIN3-derived tumour locations and arrows indicate SKOV-3/RFP-derived tumour locations. Exposure time was 100 ms for the blue-filter setting and 35 ms for the yellow-filter setting. Scale bar represents 1 cm

Advantages of multicolor imaging *in vivo* using more than one of the synthesised protease probes are:

1. Wide range of tumors that cannot be detected and characterised with a single protease activity can be captured simultaneously.
2. Tumors with a difference in protease activity profiles can be distinguished intraoperatively.
3. Some of the probes emit fluorescence in the visible region and are detectable and distinguishable from each other by the naked human eye, suggesting good translatability to clinical purposes such as intraoperative imaging.

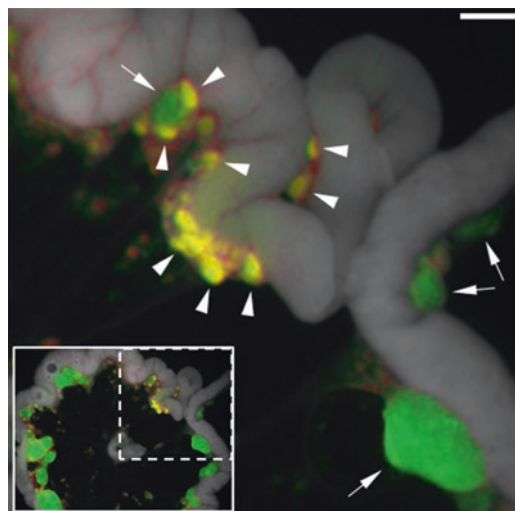


Fig. 24.8 Unmixed fluorescence images of a mouse model of dual peritoneal metastasis, at 60 min after combined intraperitoneal injection of Z-Phe-Arg-HMRG (green) and gGlu-HMJCR (red). Arrowheads indicate SHIN3-derived tumour locations and arrows indicate SKOV-3/RFP-derived tumour locations. Exposure time was 100 ms for the blue-filter setting and 35 ms for the yellow-filter setting. Scale bar represents 1 mm. Image enlarged from the dotted region of the original (inset, Fig. 24.7)

References

- Fujii T, Kamiya M, Urano Y (2014) In vivo imaging of intraperitoneally disseminated tumors in model mice by using activatable fluorescent small-molecular probes for activity of cathepsins. *Bioconjug Chem* 25:1838–1846
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70
- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144:646–674
- Iwatate RJ, Kamiya M, Urano Y (2016) Asymmetric rhodamine-based fluorescence probes for multi-colour in vivo imaging. *Chem Eur J* 22:1696–1703
- Kamiya M et al (2011) β -galactosidase fluorescence probe with improved cellular accumulation based on spirocyclized rhodol scaffold. *J Am Chem Soc* 133:12960–12963
- Miura T et al (2003) Rational design principle for modulating fluorescence properties of fluorescein-based probes by photoinduced electron transfer. *J Am Chem Soc* 125:8666–8671
- Miyata Y et al (2017) Intraoperative imaging of hepatic cancers using γ -glutamyltranspeptidase-specific fluorophore enabling real-time identification and estimation of recurrence. *Sci Rep* 7:3542
- Pompella A, De Tata V, Paolicchi A, Zunino F (2006) Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochem Pharmacol* 71:231–238
- Sakabe M et al (2013) Rational design of highly sensitive fluorescence probes for protease and glycosidase based on precisely controlled spirocyclization. *J Am Chem Soc* 135:409–414
- Shimane T et al (2016) Oral cancer intraoperative detection by topically spraying a γ -glutamyl transpeptidase-activated fluorescent probe. *Oral Oncol* 54:e16–e18

- Shinden Y et al (2016) Rapid diagnosis of lymph node metastasis in breast cancer using a new fluorescent method with γ -glutamyl hydroxymethyl rhodamine green. *Sci Rep* 6:27525
- Stewart BW, Wild CP (2014) *World cancer report 2014*. IARC Press, Lyon
- Ueo H et al (2015) Rapid intraoperative visualization of breast lesions with γ -glutamyl hydroxymethyl rhodamine green. *Sci Rep* 5:12080
- Urano Y et al (2005) Evolution of fluorescein as a platform for finely tunable fluorescence probes. *J Am Chem Soc* 127:4888–4894
- Urano Y et al (2011) Rapid cancer detection by topically spraying a gamma-glutamyltranspeptidase-activated fluorescent probe. *Sci Transl Med* 3:110ra119
- Weigelt B, Peterse JL, van't Veer LJ (2005) Breast cancer metastasis: markers and models. *Nat Rev Cancer* 5:591–602

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