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研究課題名（和文）アクロレインのインビボクリック反応性に基づく生体内合成化学治療とがんイメージング

研究課題名（英文）In vivo Click Reaction of Acrolein: Application to In vivo Synthetic Chemistry and Cancer Imaging

研究代表者
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研究成果の概要（和文）：これまでにがんの生組織を染色できる素晴らしいプローブや臨床技術が報告されているが、例えば乳がんの温存外科手術では、一回の検査に30分の長時間を必要とするにもかかわらず、未だに凍結切片のH&E染色に依る病理学的手法が専ら使用されている。がんを判別するだけでなく、様々ながんの形態を迅速に判断することが、温存手術において従来の病理学的手法を刷新する大きな壁となっている。
今回、手術中のヒト患者のがん生組織で、がんを細胞レベルで判別できる方法を実現した。術中に取り出した乳がんの生組織をそのまま我々の試薬に５分間浸し、この生組織を顕微鏡で観察するだけで様々ながんの形態を数分で識別することに初めて成功した。

研究成果の学術的意義や社会的意義
Our method has the potential to become a highly selective margin management method for live tissues. It could be used as a low-cost, and easy-to-perform method for cancer sensing during surgery. The clinical significance of our probe deserves further investigation in hospitals worldwide.

研究成果の概要（英文）：A rapid and accurate intraoperative method to diagnose the presence of breast cancer on the edge of the surgically resected tissues, is highly required. However, the current histological analysis method for determining surgical margins is time-consuming. Other groups have also reported a chemistry-based fluorescent probe that can be activated in cancer lesions. However, most of the methods rely on the time-dependent increase of fluorescence, making it difficult to use in breast-conserving surgery.
Herein, we utilized the azide-acrolein reaction-based method to discriminate breast cancer lesion from the normal breast gland. This method is the first example of an organic synthetic chemistry-based approach that can be used not only to visualize the cancer tissue but also to distinguish morphology of the resected tissue only within a few minutes. The ability to perform chemical reactions with cancer metabolites only at the desired cancer site is highly advantageous for cancer therapy.

研究分野：生体関連化学

キーワード：アクロレイン　アリールアジド　乳がん　1,3-双極子環化付加

１．研究開始当初の背景

Breast cancer is a disease with a high prevalence, affecting many women worldwide at some point during their lives. Breast-conserving surgery (BCS) is an operation that removes cancer while leaving as many healthy tissues as possible. Currently, BCS is a good option for women with early-stage breast cancer and can be combined with postoperative radiation therapy to reduce the risk of breast tumor recurrence. During BCS at the hospital, the surgeon will remove all of the cancer tissues and some areas of the surrounding healthy tissue called the surgical margins. At the same time, an on-site pathologist performs a frozen section procedure [hematoxylin and eosin (H&E) staining] for morphological analysis of the surgical margins, which usually takes at least 30 – 60 minutes, while the patient is still under anesthesia, and the surgical cut is still open (**Fig. 1**). If the pathologist finds that the surgical margin is a "normal breast gland" (NBG), it is said to have negative margins. However, if "ductal carcinoma in situ" (DCIS) or "invasive ductal carcinoma" (IDC) is found, it is said to have positive margins. The presence of positive margins means that some cancer cells may still be in the patient's breast. Thus, the surgeon may need to go back to the operation room and remove more tissues. This conventional method has the potential to lower the rates of positive margins. However, the method cannot be generally applied to current BCS in hospitals worldwide due to the laborious and time-intensive procedures. Moreover, the number of hospitals that perform breast cancer surgery exceeds the number of board-certified pathologists, eliminating the possibility for expert intraoperative consultation in many cases.

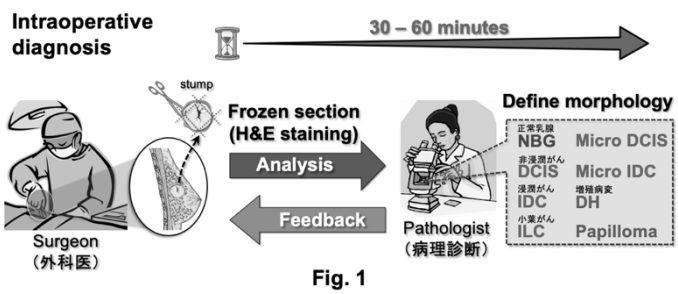

Fig. 1

２．研究の目的

Recently, we discovered a 1,3-dipolar cycloaddition reaction between aryl azide and acrolein, which proceeds without a catalyst to give a 4-formyl-1,2,3-triazoline derivative. The reaction proceeds with high reactivity and selectivity even under physiological conditions. We have successfully utilized the click reaction as a simple and robust method for detecting acrolein generated by live cells.

Herein, we utilized the azide-acrolein click reaction-based method to discriminate breast cancer lesions from the normal breast gland, which were resected from breast cancer patients. This method is the first example of an organic synthetic chemistry-based approach that can be used not only to visualize the cancer tissue but also to distinguish the morphology of the resected tissue only within a few minutes. It has a potential clinical application for breast-conserving surgery. Furthermore, the ability to perform chemical reactions with cancer metabolites only at the desired cancer site is highly advantageous for cancer therapy. If it succeeds, this method can be readily used during surgery, even in a small hospital, where expensive equipment and pathologist is not always available.

Acrolein, the most reactive α,β-unsaturated aldehyde, has long been known as a critical biomarker associated with a range of disorders related to oxidative stresses, including cancers and Alzheimer's disease. Acrolein is produced through the enzymatic oxidation of polyamines and is also generated during reactive oxygen species (ROS)-mediated oxidation of highly unsaturated lipids. It is sometimes generated on a few hundred μM scales in oxidatively stressed cells. It is more toxic to cells than ROS, such as hydrogen peroxide (H_2O_2) or hydroxyl radical (•OH), the major oxidative stress factors associated with a variety of disorders.

Previously, we described the 1,3-dipolar cycloaddition between phenyl azide **1** and acrolein, which proceeded under physiological conditions, even without a catalyst, to give the 4-formyl-1,2,3-triazoline derivative **2** (**Fig. 2a**). The reaction is highly chemoselective to acrolein. Any clicked products could not be observed by the reaction with α- or β- substituted acrolein (e.g., methacrolein, crotonaldehyde, *trans*-2-octenal) under the physiological conditions. By attaching TAMRA fluorophore to the phenyl azide, namely click-to-sense probe (**CTS probe, Fig. 2b**), the reaction could be used selectively and sensitively to detect acrolein in live cells even at the nM level.

We also revealed the mechanism for visualization of acrolein in live cells (**Fig. 2b**). We found that the cellular uptake of the **CTS probe** is mediated by endocytosis. The TAMRA scaffold is essential for internalizing the probe into the cells. It is worth noting that the **CTS probe** could continuously shuttle between the inside and outside of cells through transporter-mediated mechanisms. If the **CTS probe** encounters the intra-cellular acrolein, the 1,3-dipolar cycloaddition reaction occurs to produce a 4-formyl-1,2,3-triazoline derivative that decomposed into a diazo compound and reacted with the nearest organelle to anchor the fluorophore via covalent attachment. Therefore, cancer could be labeled and imaged at the cellular level.

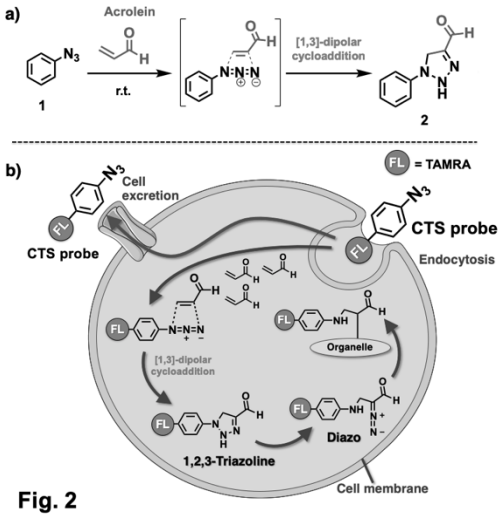

Fig. 2

We demonstrated the feasibility of using this click-to-sense (CTS) method to determine the acrolein levels in various human cell lines (**Fig. 3**). The procedure required two simple steps; first, the cells were treated with a **CTS probe**. Second, the total fluorescence intensity, which was proportional to the acrolein concentration generated by the cells, was measured.

Eleven cell lines investigated in the study include eight cancer cells [PC3 (human prostate cancer), HeLa S3 (human cervix cancer), A549 (human lung cancer), MCF7 (human breast cancer), HT29 (human colon cancer), BxPC3 (human pancreatic cancer), MDA-MB-231 (human breast cancer), and SKBR3 (human breast cancer cells)] and three normal cells [MCF10A (human normal mammary cell), HUVEC (human umbilical vein endothelial

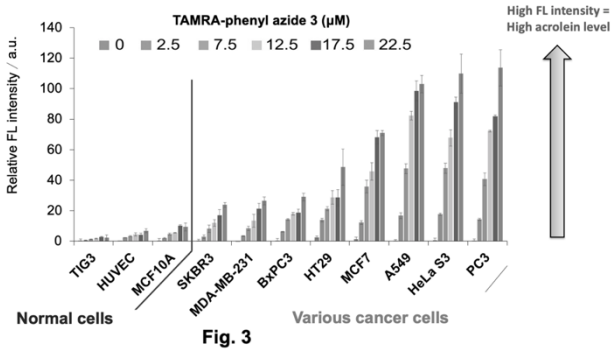

Fig. 3

cells), and TIG3 (human normal diploid cell)]. The higher fluorescence intensity means a higher amount of acrolein released by the cells. Hence, we have shown that cancer cells express a high acrolein level, whereas normal cells only express a negligible amount of acrolein (**Fig. 3**). In other words, cellular acrolein could be used as a new cancer marker.

Based on the observation that acrolein is produced by cancer cells in significant amounts, we hypothesized that the **CTS probe** could selectively and sensitively detect acrolein in cancerous tissues. Herein, we utilized **the CTS probe** to detect acrolein in breast cancer tissues resected from breast cancer patients (**Fig. 4**). The results surpassed our expectations. Our method overcomes many of the limitations of other techniques so far reported. Unlike other methods, it can discriminate, in a clear-cut manner, cancer [invasive ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS)] from normal breast gland (NBG) and ductal hyperplasia (DH), and sensitively visualize cancer morphology and localization in the resection stump.

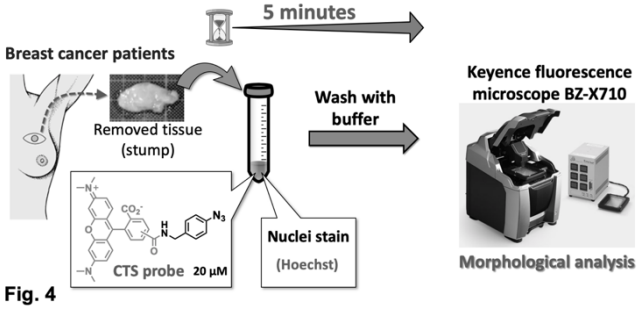

Fig. 4

４．研究成果

We performed analysis on 30 cancer (20 IDC and 10 DCIS) tissues, 30 NBG tissues, and 5 DH tissues from 30 breast cancer patients who underwent a breast surgery at Osaka University Hospital using the double fluorescence staining method (**CTS probe** and Hoechst, **Fig. 4**). The live tissues were cut by Tissue Matrix Chamber to create a flat surface, dipped into 20 μM solution of the **CTS probe** for 5 minutes, and rinsed with buffer. The resulting tissues were then directly analyzed by Keyence BZ-X710, equipped with an optical sectioning algorithm system to obtain both gross pictures and double fluorescence staining images. The Keyence sectioning algorithm allows the user to obtain clear images without fluorescence blurring, in a way comparable to those captured on a laser confocal microscope, but in a fraction of the time and without the damaging effects.

Representative images are shown in **Figure 5a-left**. In both the gross and fluorescent images, the breast cancer tissues (IDC or DCIS) were more highly stained by our **CTS probe** than NBG tissues. The statistical significance of the tumor selectivity is shown in **Figure 5a-right**. The mean fluorescence intensity of breast cancers (IDC or DCIS) was significantly higher than that of NBG tissue in a statistically significant dose-dependent manner. When we optimized the dividable threshold value of fluorescence intensity, 97% of sensitivity and 97 % specificity were obtained for binary classification between breast cancers and NBG. Notably, the **CTS probe** labeled the breast cancer tissues with similar mean fluorescence intensity, regardless of IDC subtypes such as estrogen receptor (ER) or human epidermal growth factor receptor 2 (HER2) status, so as of DCIS subtypes for different ER status.

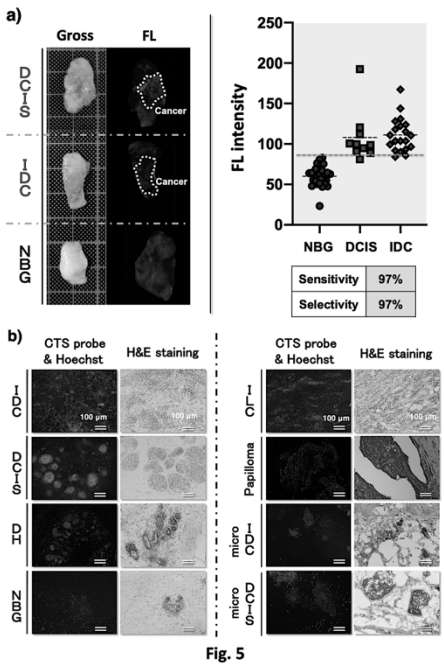

Fig. 5

By magnifying the fluorescence-labeled IDC and DCIS images (200×), the morphology of IDC and DCIS could be imaged (**Fig. 5b**). The pathologists diagnosed and compared the double fluorescence staining images (**CTS probe** and Hoechst) with the H&E staining images of the same tissue samples in anonymized form. It should be noted that the fluorescent images of cancers are in good agreement with those of H&E staining. Thus, this marks the first successful imaging of cancer morphology at the live tissue level outside of conventional pathology methods.

Our findings indicated that acrolein detection in cancerous tissues using the **CTS probe** was feasible based on the fluorescence images. This notable feature of the **CTS probe**, which enables the visualization of cancer morphology, comes from its ability to selectively label the cellular contents only in cancer cells within live tissues (**Fig. 2b**). Because only the cancer cells were labeled selectively by **the CTS probe**, this chemistry-based diagnosis method might be helpful for rapid intraoperative diagnosis during BCS at the hospital.

With this promising method, live tissue morphology in patients with breast cancer can be easily identified, providing future support for BCS. Our click-to-sense (CTS) method, which only requires 5 minutes, has the potential to become a new, highly selective margin management method for live tissues; it could be used as a discriminative, low-cost, and easy-to-perform method for cancer sensing during surgery. The technique will be confirmed in a prospective clinical study, including intraoperative assessment of resection stumps in patients with breast cancer (**Fig. 6**). The clinical significance of our probe in evaluating morphological and pathologic features deserves further investigation in hospitals worldwide.

Fig. 6

It would be useful to consider applying automatic deep learning and artificial intelligence (AI) algorithms. AI has shown potential usefulness as a diagnostic tool in pathologic diagnosis. Once efficient intraoperative techniques have been developed, then AI can automatically and rapidly evaluate cancer margins. Of note, our method could be used to diagnose cancer tissues during BCS faster than the conventional H&E method. Combining this method with AI would lead to the next generation of real-time intraoperative assessment.

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２．論文標題 Tetramethylrhodamine is an essential scaffold of azide probe in detecting cellular acrolein	５．発行年 2019年
３．雑誌名 Bioorganic & Medicinal Chemistry	６．最初と最後の頁 2228～2234
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bmc.2019.04.026	査読の有無 有
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２．論文標題 Facile Access to Optically Active 2,6‐Dialkyl‐1,5‐Diazacyclooctanes	５．発行年 2019年
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３．雑誌名 J. Struct. Chem.	６．最初と最後の頁 -
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２．論文標題 Emerging Technologies for Real‐Time Intraoperative Margin Assessment in Future Breast‐Conserving Surgery	５．発行年 2020年
３．雑誌名 Advanced Science	６．最初と最後の頁 1901519～1901519
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2．論文標題 Expanding the Applicability of the Metal Labeling of Biomolecules by the RIKEN Click Reaction: A Case Study with Gallium-68 Positron Emission Tomography	5．発行年 2018年
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2．論文標題 In vitro and in vivo cancer cell apoptosis triggered by competitive binding of Cinchona alkaloids to the RING domain of TRAF6	5．発行年 2018年
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2．論文標題 Cascade Reaction in Human Live Tissue Allows Clinically Applicable Diagnosis of Breast Cancer Morphology	5．発行年 2019年
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掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/advs.201801479	査読の有無 有
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2．論文標題 Cycloaddition Reactions of N-Alkyl-α,β-unsaturated Imines: Facile Preparation of Azaheterocycles for Synthesis and Biological Applications	5．発行年 2018年
3．雑誌名 HETEROCYCLES	6．最初と最後の頁 668～685
掲載論文のDOI（デジタルオブジェクト識別子） 10.3987/REV-18-SR(T)4	査読の有無 有
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1．著者名 Ambara R. Pradipta, Elena Saigitbatalova, Almira Kurbangalieva, Katsunori Tanaka	4．巻 48
2．論文標題 Poster Abstract	5．発行年 2018年
3．雑誌名 European Journal of Clinical Investigation	6．最初と最後の頁 219
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2．論文標題 Poster Abstracts I	5．発行年 2019年
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〔図書〕　計0件

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７．科研費を使用して開催した国際研究集会

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８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関