別紙様式第４

学　位　論　文　要　旨

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[論文題名]

A nucleoside derivative 5-vinyluridine (VrU) for imaging RNA in cells and animals

ヌクレオシド RNA 誘導体 5-ビニルウリジン（VrU）の創製及び細胞・動物 RNA のイメージング

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（雑誌の表紙に採用）

[要　旨]

Ribonucleic acid (RNA) is a polymeric molecule essential in various biological roles in coding, decoding, regulation and expression of genes. Imaging of RNA and DNA as a basic technology has many interesting applications in genetics, gene expression and cell biology. This technology can help us better study the structure and function of DNA and RNA molecules in cells (***Science 1996, 273, 494-497***). Imaging RNA and DNA is also of importance for chromosomal analysis and clinical genetic diseases.

In previous studies, 5-ethynyl-2'-deoxyuridine (EdU) and 5-ethynyluridine (EU) were used to label DNA and RNA in cells respectively, with azide–alkyne click reaction in cells (***Proc. Natl. Acad. Sci. U.S.A. 2008, 105, 15779-15784***). Then, Luedtke et al. reported using the Diels–Alder reaction for imaging DNA with 5- vinyl-2'-deoxyuridine (VdU). 5-vinyluridine (VrU) was utilized for modifying RNA by solid-phase method and by using RNA polymerase *in vitro* (***Angew. Chem. Int. Ed. 2014, 53, 9168-9172***). Not long ago, Kubota et al. reported that VrU can be used to labeling RNA in cell (***ACS Chem. Biol. 2019, 14, 1698-1707***).

Although advances in labeling DNA and RNA have been made by bioorthogonal chemistry in previous reports, the use of chemical methods to simultaneously visualize DNA and RNA in two colors at the single cell level still remains challenging. Because of the same alkyne groups in EdU and EU, they will react with fluorescent azides simultaneously. Therefore, they cannot discriminate DNA and RNA by different colors in a single cell (***Nucleic Acids Res. 2016, 44,***

e16). VrU-labeled cellular RNA can be detected quickly with a functionalized tetrazine bearing a biotin moiety using Diels–Alder reaction. DNA can be imaged using EdU through the azide–alkyne click reaction. Thus, the combination of EdU and VrU can be employed to image DNA and RNA with different colors at single cells level. For living cells or animals' experiments, the catalytic toxicity will affect cell morphology, so VdU and VrU using Diels–Alder reaction is more suitable for labelling in living systems as compared with EdU and EU using the Cu(I)-catalysed azido–alkyne cycloaddition (CuAAC).

In this study, we used the VrU and EdU to simultaneously observe DNA and RNA at single-cell resolution, we further extended this approach to observe DNA and RNA behaviors in several basic of cell division and for tumor imaging in living mice. We show that VrU is incorporated into RNA. VrU-labeled cellular RNA can be detected quickly with a functionalized tetrazine bearing a biotin moiety (Biotin-tetrazine) using Diels–Alder reaction. We also perform BODIPY-tetrazine as a light-up method for imaging RNA. We used the nucleoside derivative to simultaneously visualize DNA and RNA in dual-color at the single-cell level by combining EdU. We further employed the two derivatives (VrU and EdU) to simultaneously observe RNA and chromosome DNA behavior in several basic stages of cell division. We also found that the VrU can be used to image tumor in live mice. The nucleoside derivative therefore provides an effective approach to introduce a bioorthogonal functional group into RNA for diverse applications.

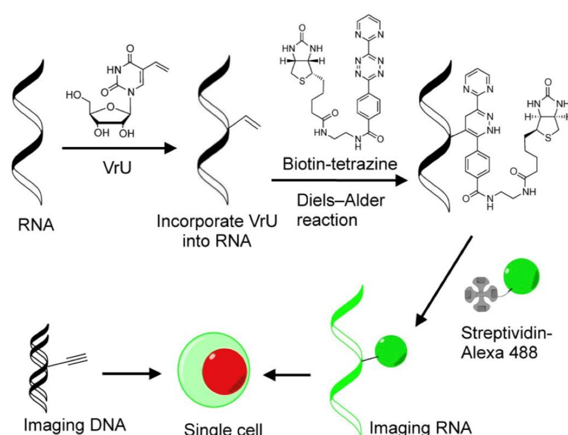

Figure 1. Schematic of imaging RNA by Diels–Alder reaction of VrU and biotin-tetrazine and imaging DNA by EdU–azide cycloaddition in single cell. Structures of VrU and biotin-tetrazine are shown. For imaging VrU in cellular RNA, streptavidin-Alexa 488 (green) was used for revealing fluorescence signal.

At first, we characterize the chemical reactivity of VrU. VrU and biotin-tetrazine were incubated in 1:1 for time-dependent LC-MS analysis. After 48 h mixing, we found that the peaks of VrU and biontin-tetrazine decreased, and new peaks appeared. The new peaks

suggested that VrU can react with biotin-tetrazine, and the intensity of the new peaks demonstrated an overall yield about 55%. Then we also proved there is no cross reaction between azide–alkyne click reaction and Diels–Alder reaction by HPLC under the appropriate reaction conditions.

Next, we apply this method to image RNA in cells. HeLa cells were incubated with VrU for 3 h, reacted with biotin-tetrazine, and finally incubated with streptavidin-Alexa Fluor 488. As shown in Figure 2A, HeLa cells showed high fluorescence intensity with VrU staining, and cells lacking VrU labeling showed almost no fluorescence under the same reaction conditions (Figure 2B). Not only that, we successful observed dual-color imaging of DNA and RNA in single cells, using VrU–tetrazine reaction in RNA, in combination with EdU–azide cycloaddition in DNA (Figure 2f and 2J). We performed the control reactions in the presence of RNA inhibitor actinomycin D and RNase to demonstrate that VrU labels cellular RNA. We further performed a time course of VrU imaging to detect the staining efficiency of VrU in cells. The results from flow cytometry shown that about 76% cells having fluorescence signal after 10 min incubating with VrU. After 30min incubating with VrU, the fluorescence intensity became stronger, and more than 90% of cells could be detected fluorescence. We also performed a concentration-dependence experiment. The flow cytometry indicated that about 70 % cells could be detected fluorescence signals from 50 μM concentration. From 200 μM incubating concentration, over 90 % cells could be observed fluorescence signals and have higher fluorescence intensity.

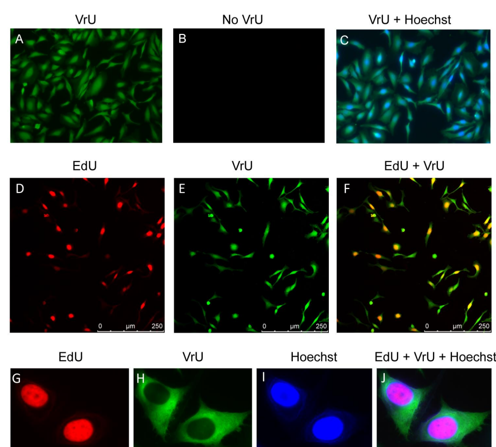

Figure 2. Incorporation of VrU into cellular RNA. (A) HeLa cells incubated with 1 mM VrU, 300 μM biotin-tetrazine and streptavidin-Alexa Fluor 488 (green). (B) Same condition of incubating cell with (A) but absence of the VrU. (C) HeLa cell imaging by VrU and staining DNA by Hoechst. (D) Cellular DNA is incorporated EdU and stained by Alexa594-azide (red). (E) Cellular RNA is incorporated VrU and stained by streptavidin-Alexa Fluor 488 (green). (F) Overlay of (D) and (E) images. (G) ~ (J) Cell imaging by EdU, VrU and Hoechst.

The stability of VrU-labeled transcripts was determined by VrU pulse–chase in cells. After a short time pulse the cell imaging decrease quickly in the process of chasing. But after a longer pulse, the signal remains strong even 24 h into the chase, these indicated the labeling of stable RNA species.

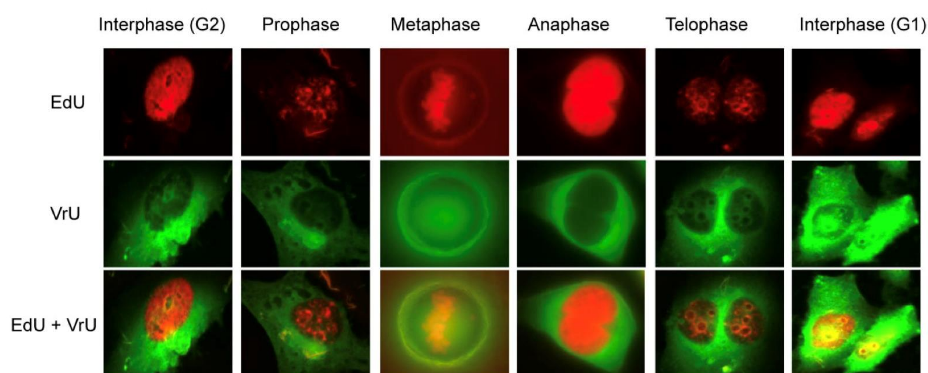

Figure 3. Observation of DNA and RNA behaviors in several basic stages of cell division. DNA is stained by Alexea594-azide (red). RNA is stained by streprtavidin-Alexa Fluor488 (green).

Then we successful observe DNA and RNA behaviors in several basic stages of cell division（Figure 3）. We also proved the changes of DNA and RNA in cells during different periods. We found that in interphase labeled RNA can be clearly detected in nucleus and cytoplasm; during mitosis as prophase and anaphase, RNA fluorescence is a weakness in nucleus. These suggested that the nuclear RNA synthesis is associated with cell division.

We further used the nucleoside derivative for fluorescence imaging of tumor in live mice. To image cancer in vivo, we developed a light-up (turn on) reporter strategy, using BODIPY–tetrazine derivative as the pro-fluorophore that can produce a strong fluorescence in a Diels-Alder reaction. We clearly observed green color in an emission (527/30 nm) filter in living cells. Subsequently, VrU was injected into nude mice bearing LS—180 tumor. As shown in Figure 4, the subcutaneous LS—180 tumor could be clearly distinguished from the control mice at postinjection of BODIPY–tetrazine. Imaging of the excised tumor showed a higher fluorescence intensity. These experiments demonstrated the cancer-selective labeling capability of the light-up strategy in vivo.

In summary, we demonstrated that the VrU reagent allows for quick and sensitive RNA imaging in vitro and in vivo. We are able to simultaneously and clearly image DNA and RNA in mammalian cells at the single-cell resolution. This approach can be extended to observe DNA and RNA behaviors in several basic stages of cell division. Moreover, the nucleoside derivative can perform the biorthogonal reaction in tumor-bearing mice for tumor-specific

imaging.

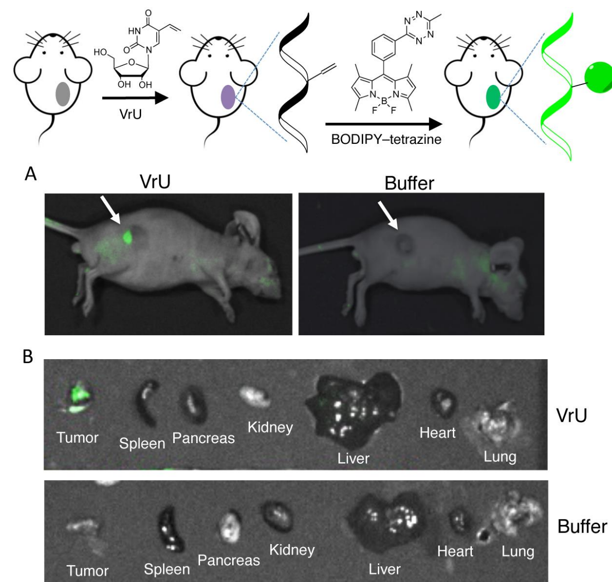

Figure 4. In vivo labeling of mice bearing tumor with VrU and subsequent cancer targeting via a light-up strategy using BODIPY–tetrazine derivative in a Diels–Alder reaction. (A) In vivo fluorescence imaging of mice from buffer and VrU groups. Tumors are shown by arrows. (B) Ex vivo fluorescence imaging of tissues excised from mice treated with VrU or PBS buffer.

備考　論文要旨は、和文にあっては２，000 字程度、英文にあっては１，200 語程度