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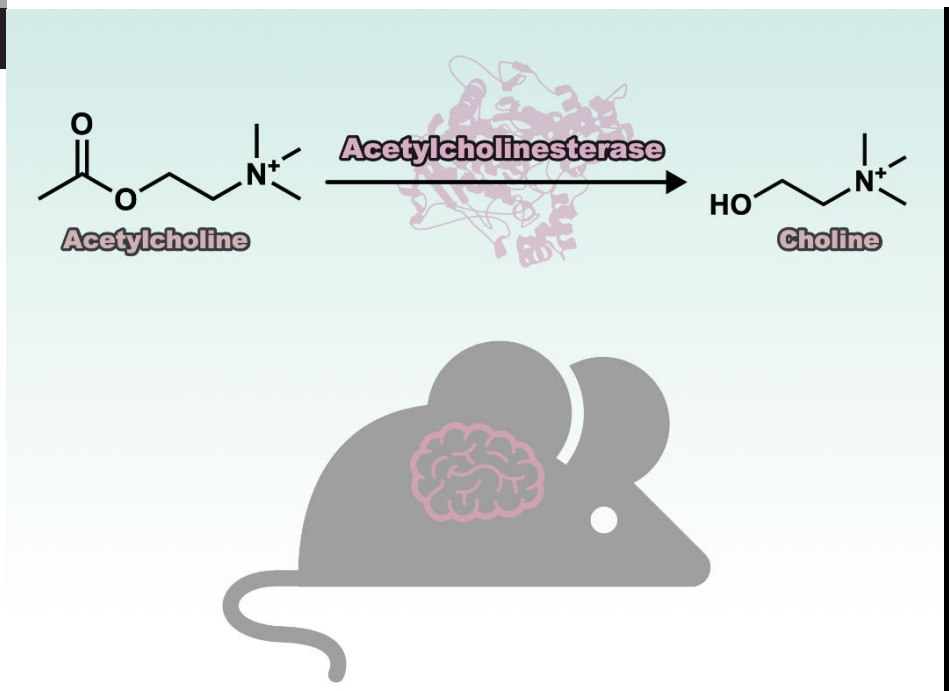
Application Note

No. 68

Life Science

Enzyme Histochemistry Using Mass Spectrometry Imaging

Shuichi Shimma[1, 2, 3], Emi Takeo[1], Kaoru Nakagawa, Takushi Yamamoto, and Eiichiro Fukusaki[1, 2, 3]



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■ Abstract

Enzymatic reactions are commonly detected by reacting a substrate and enzyme, using the reaction products in a subsequent reaction to produce a color response, then measuring the absorbance. Existing methods of detecting enzymatic reactions require both the primary reaction between the substrate and enzyme and the secondary reaction for a color response. A new method of detecting enzymatic reactions forgoes the secondary reaction and instead detects primary reaction products with a mass spectrometer. Applying this technique on the surface of a tissue sample also allows for visualization of enzyme activity. This Application Note describes performing this new enzyme histochemistry technique in an example that uses iMScope™, an instrument capable of performing mass spectrometry imaging at high resolution.

1. Introduction

The distribution of enzymes within tissue is commonly determined by immunohistochemistry (IHC). Though IHC can visualize the location of enzyme proteins, it cannot distinguish between active and inactive enzymes. Enzyme histochemistry is a long-established technique used to visualize the distribution of enzyme activity, otherwise unachievable by IHC.[1),2)] Because enzyme histochemistry uses chemical reactions dependent on enzyme activity on the surface of tissue sections, it can identify enzyme activity and also visualize the strength of that activity. Visualization is normally achieved by applying the substrate to a tissue section, where it reacts with endogenous enzymes and the reaction products are used to produce a color response via another reaction. Using this method, each reaction that produces color does so in response to a single compound, hence visualizing multiple compounds requires multiple color-producing reactions. As a result, visualizing a distribution of enzyme activity by this method is often not a simple process of adding substrate to a tissue section. As an alternative to the color-producing reaction step of conventional enzyme histochemistry, this study investigated using mass spectrometry imaging to detect the products of enzymatic reactions directly in mouse brain sections and whole *Drosophila* sections.[3)]

2. Experiments

This study attempted to visualize the distribution of acetylcholine esterase (AChE) activity in wild-type mouse brain sections and whole wild-type *Drosophila* sections. AChE is an enzyme that catalyzes the breakdown of the substrate acetylcholine into choline and acetic acid. Accordingly, acetylcholine was applied to the surface of each tissue sample and the degradation product choline was detected. Choline produced by this reaction was differentiated from endogenous choline by using deuterium-labeled acetylcholine-d9 as the substrate and detecting choline-d9 (Fig. 1). The substrate was sprayed manually onto tissue sections with an airbrush. This study also examined reaction times and techniques for semiquantitative analysis.

[1] Department of Biotechnology, Graduate School of Engineering, Osaka University
[2] Osaka University Shimadzu Omics Innovation Research Laboratories
[3] Institute for Open and Transdisciplinary Research Initiatives, Osaka University

α-Cyano-4-hydroxycinnamic acid (α-CHCA, Sigma-Aldrich) was used as the matrix, and the matrix was applied using a two-step vapor deposition method (Patent No.: 6153139)[4] that combined vapor deposition using an iMLayer™ matrix vapor deposition system (Fig. 2) and manual spraying of α-CHCA solution.

MSI measurements were performed using an iMScope imaging mass microscope (Fig. 3) and data were analyzed using IMAGEREVEAL™ MS mass spectrometry imaging data analysis software (Fig. 4). Data were acquired using an iMScope configured with the analysis parameters shown in Table 1.

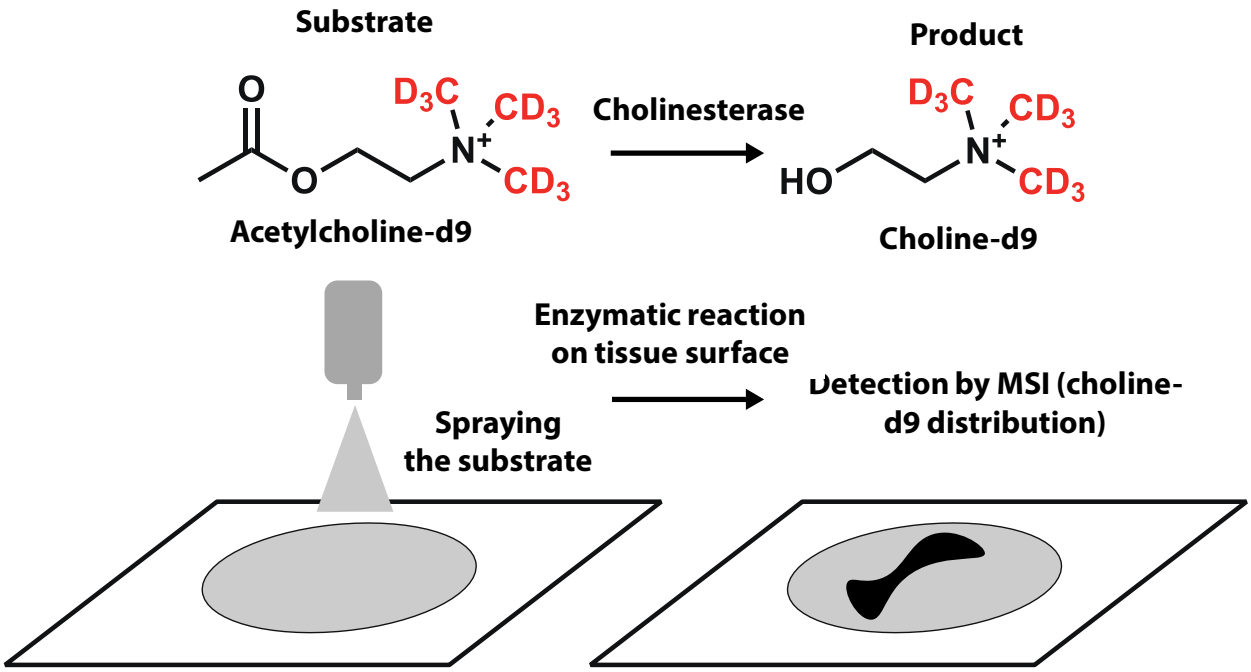

Fig. 1 Outline of Enzyme Histochemistry with MSI
A labeled substrate is applied to the sample surface and products of the enzymatic reaction are detected by mass spectrometry and visualized.

Fig. 2 iMLayer™ Matrix Vapor Deposition System

Fig. 3 iMScope™ QT Imaging Mass Microscope

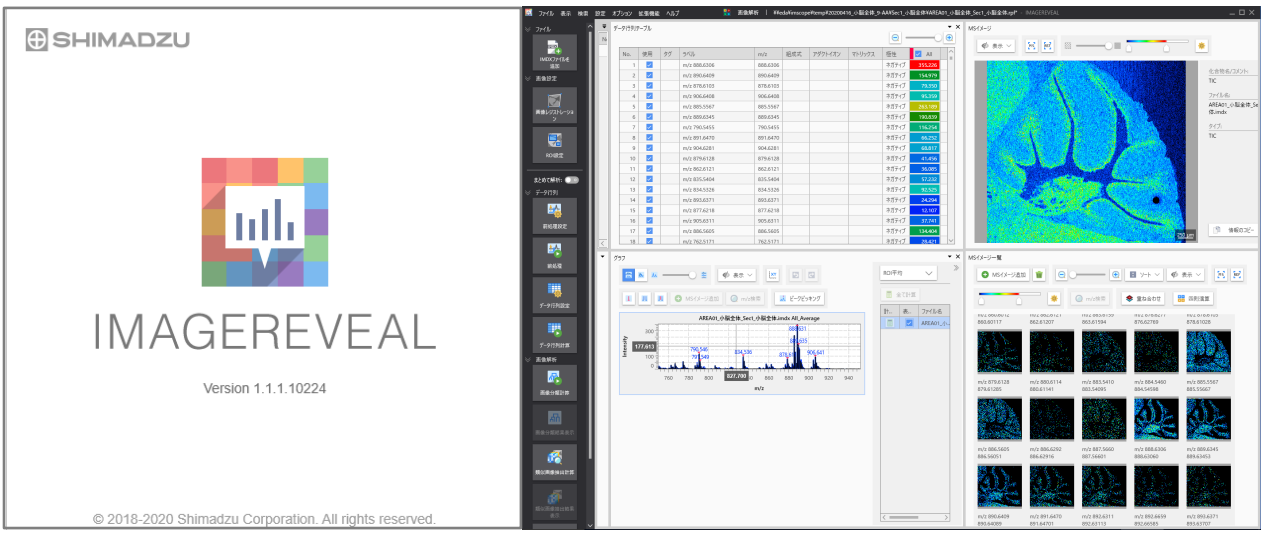

Fig. 4 IMAGEREVEAL™ MS Mass Spectrometry Imaging Data Analysis Software

Table 1 MSI Analysis Parameters

MS Analysis Conditions	
Ion Type	Positive-ion mode
m/z Measurement Range	100-160
Averaged Acquisitions	1
Sample Voltage	3.50 kV
Detector Voltage	2.10 kV
No. of MS Steps	1
Laser Irradiation Conditions	
Irradiation Count	100
Repetition Frequency	1000 Hz
Laser Diameter Settings	0 (approx. 5 µm) or 1 (approx. 10 µm)
Laser Intensity	20 or 45

$$\text{Conversion rate (\%)} = 100 \times \frac{I_{\text{choline-d9}}}{I_{\text{ACh-d9}} + I_{\text{choline-d9}}} \quad \ldots(1)$$

$$\text{Relative ChE activity (au)} = 100 \times \frac{\varepsilon I_{\text{choline-d9}}}{I_{\text{ACh-d9}} + \varepsilon I_{\text{choline-d9}}} \quad \ldots(2)$$

I : Intensity
$\varepsilon = 3$

Fig. 5 Conversion Rate Equation and Enzyme Activity Equation

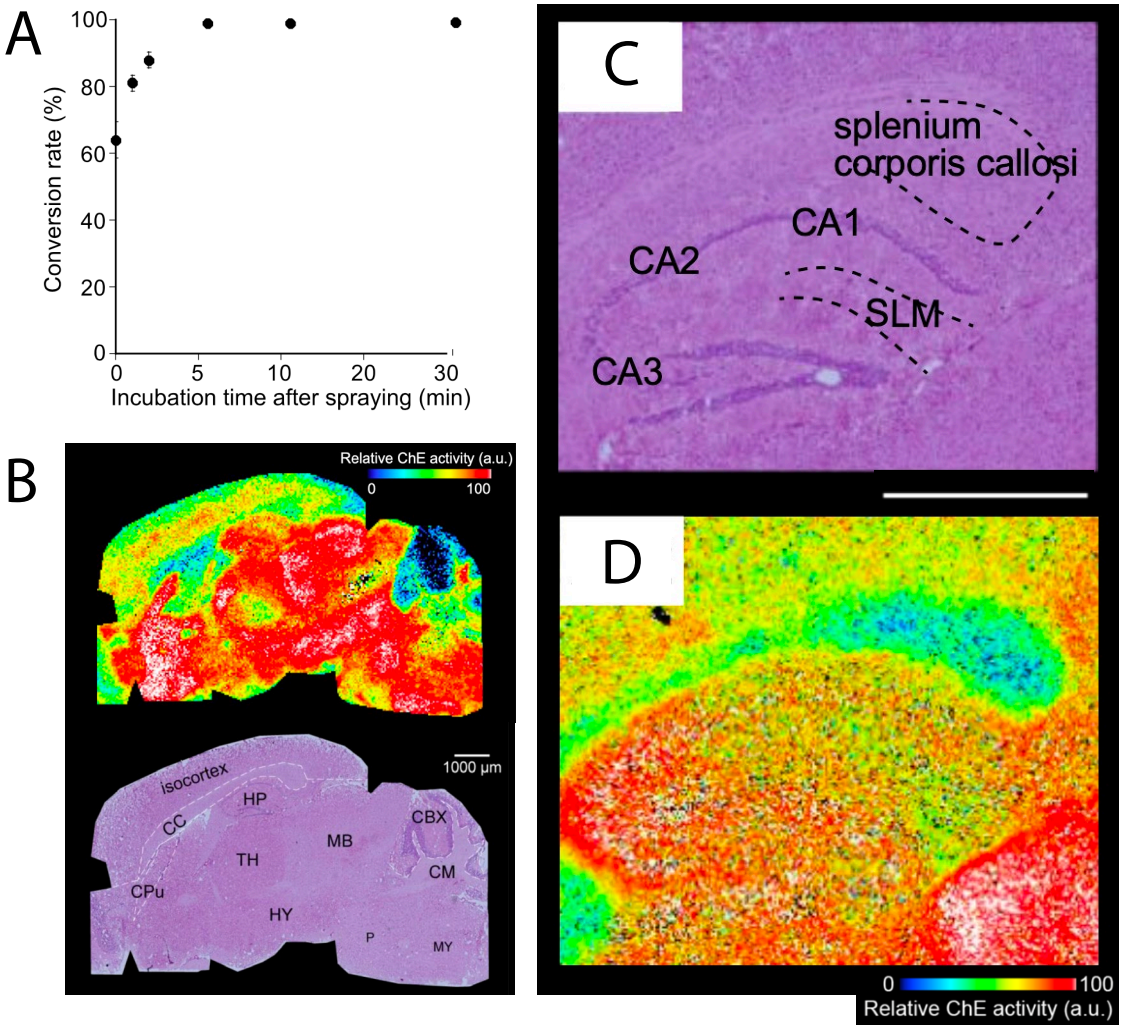

Fig. 6 (A) Percentage Converted Substrate Plotted Against Enzymatic Reaction Time on Tissue
Taking the spraying of substrate as 0 minutes, results show that all acetylcholine-d9 (substrate) was converted to choline-d9 within 5 minutes.

(B) Distribution of Cholinesterase Activity in Mouse Brain Tissue
Compared to HE staining, results show high activity in the corpus striatum (CPu), hippocampus (HP), and hypothalamus (TH) and low activity in the corpus callosum (CC) and cerebellar cortex (CBX).

(C, D) HE Staining and High-Resolution Imaging of Activity in Mouse Hippocampus
Shows high enzyme activity in CA3 region. Scale bar: 1 mm

3. Results and Discussion

Calculating substrate conversion rates using the equation in Fig. 5 (1) and plotting conversion rates against reaction times shows that acetylcholine-d9 was quickly decomposed into choline-d9 after spraying onto the test surface, and conversion plateaued and acetylcholine-d9 was depleted within 5 minutes of reaction time (Fig. 6A). These results confirm that 5 minutes is enough reaction time to measure enzyme activity. Given that matrix effects associated with tissue location pose issues for semiquantitative analysis, the equation in Fig. 5 (2) was conceived as a method of standardization that accounts for the ionization efficiencies of acetylcholine-d9 and choline-d9.

IMAGEREVEAL MS mass spectrometry imaging data analysis software was used to create MS images of acetylcholine-d9 and choline-d9 from peaks at *m/z* 155.17 and *m/z* 113.16. Using the four basic arithmetic operations available in IMAGEREVEAL MS, these MS images were inserted into equation (2) to obtain images that show the distribution of cholinesterase activity (Fig. 6B and Fig. 6D). These images revealed high AChE activity in the corpus striatum (CPu), hippocampus (HP), and hypothalamus (TH), and low AChE activity in the corpus callosum (CC) and cerebellar cortex (CBX) (Fig. 6B).

When these results were compared with conventional enzyme histochemistry, it revealed an extremely good match and established proof of concept for this technique. The high spatial resolution afforded by imaging with the iMScope was also used to visualize enzyme activity in the hippocampal region of the brain (Fig. 6C, 6D).

Because mammals produce butyrylcholinesterase (BuChE) in addition to AChE, an attempt was made to visualize the activity distribution of each of these cholinesterases. BuChE converts acetylcholine and various other choline esters into choline. Using MSI, the distribution of AChE-specific activity was visualized by applying the substrate acetylcholine together with tetraisopropyl pyrophosphoramide (iso-OMPA), an inhibitor of BuChE. The distribution of BuChE-specific activity was also visualized by analyzing serial tissue sections and applying the substrate acetylcholine together with galantamine, an inhibitor of AChE activity. These experiments showed that enzyme activity detected in the corpus callosum without inhibitors was largely inhibited by iso-OMPA, suggesting that much of the cholinesterase activity in the corpus callosum was due to BuChE (Fig. 7A).

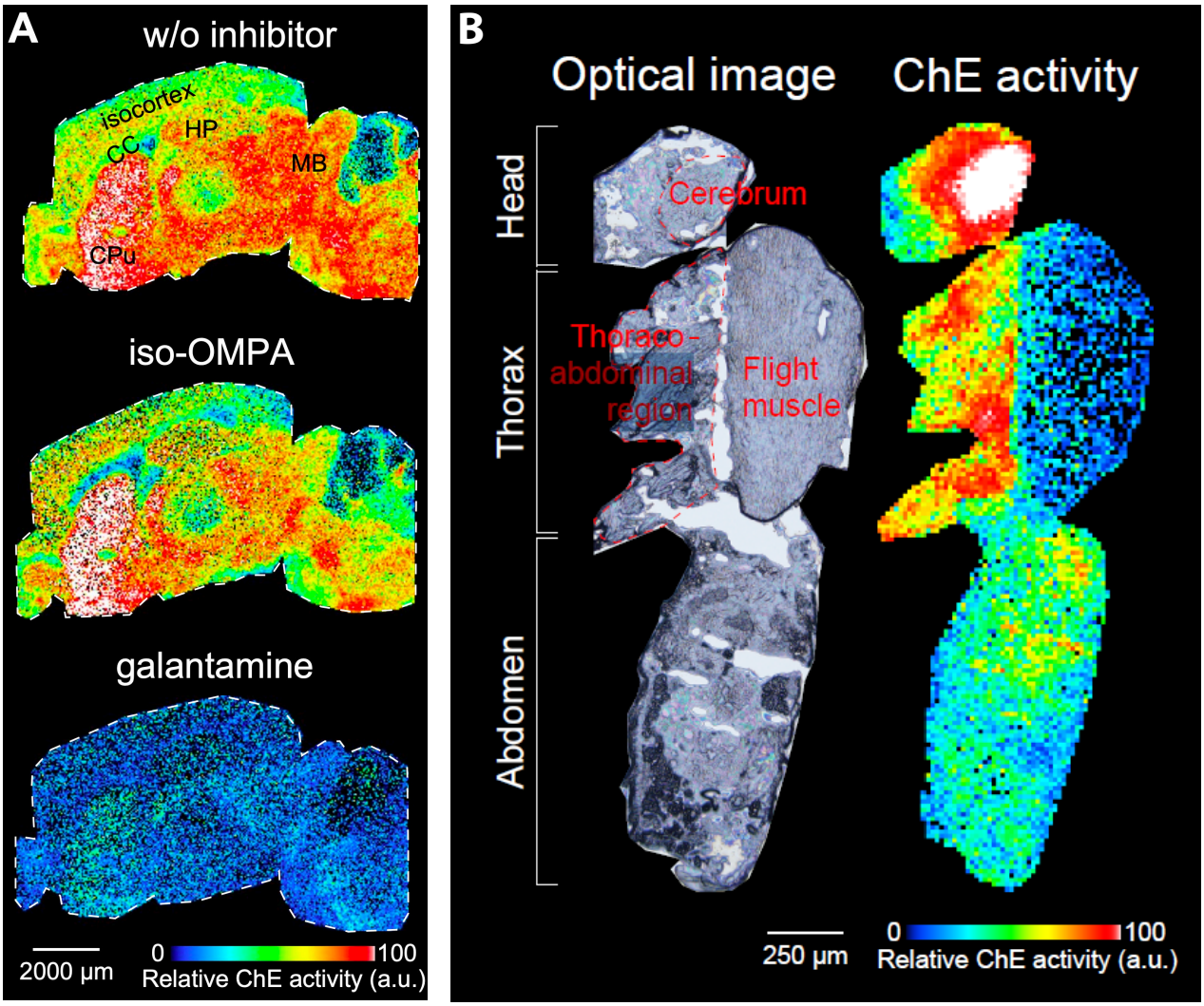

Fig. 7 Enzyme Activity Visualized with Inhibitors in a Mouse Brain Section, and MSI of Cholinesterase Activity Distribution in a Whole *Drosophila* Section

(A) Enzyme Activity Visualized with Inhibitors
iso-OMPA inhibits butyrylcholinesterase activity and allows the specific detection of acetylcholinesterase activity.
Galantamine inhibits acetylcholinesterase activity and allows the specific detection of butyrylcholinesterase activity.

(B) Distribution of Cholinesterase Activity in *Drosophila*
Although *Drosophila* is of a different phylum, this technique was applicable and revealed enzyme activity in the brain and the thoracoabdominal region. Particularly in the thoracoabdominal region, soluble enzyme activity was detected, showing this technique can provide visualization that is difficult to obtain with conventional enzyme histochemistry.

Therefore, applying a stable isotope substrate to the sample surface with inhibitors has been demonstrated as a more precise enzyme histochemical technique.

This technique can even be used on *Drosophila*, an animal of a different phylum. As shown in Fig. 7B, ChE activity was spread unevenly through *Drosophila* with extremely high ChE activity in the cerebrum and high ChE activity in the thoracoabdominal region. The particularly high level of enzyme activity in the head is consistent with prior reports[5] and suggests activity is due to AChE in cholinergic nerves of cephalic ganglia in the central nervous system. By contrast, ChE activity in the thoracoabdominal region is probably not due to AChE in the central nervous system. Reports show AChE is also present in blood lymphs in addition to the nervous system[6] and Zador et al. note the existence of soluble AChE with a structure that differs from membrane-bound AChE found on nerve cells[7]. The AChE activity revealed in the thoracoabdominal region is consistent with these prior reports and demonstrates the validity of ChE activity localization shown by this technique.

4. Conclusion

This Application Note describes a new MSI-based enzyme histochemistry and shows MSI gives a semiquantitative distribution of enzyme activity without using color-developing reactions. This technique was also used on *Drosophila* sections and shown to be effective in visualizing the activity of both membrane-bound AChE and soluble AChE. The distribution of soluble enzyme activity is particularly difficult to obtain by conventional methods and this attests to the superiority of the new technique described in this Application Note. We intend to develop more visualization methods for other enzymes (not only hydrolases but transferases, etc.).

Acknowledgments

We are sincerely grateful to Professor Masamitsu Yamaguchi of the Chromosome Engineering Laboratory, Department of Applied Biology, Kyoto Institute of Technology (at the time of this study) for supplying the *Drosophila* samples.

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First Edition: Nov. 2021

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