Introduction

ISHpalette™ Short hairpin amplifier is a fluorescent hairpin DNA reagent developed as an enhanced version of the traditional *in situ* HCR method for detecting target molecules in tissue sections.

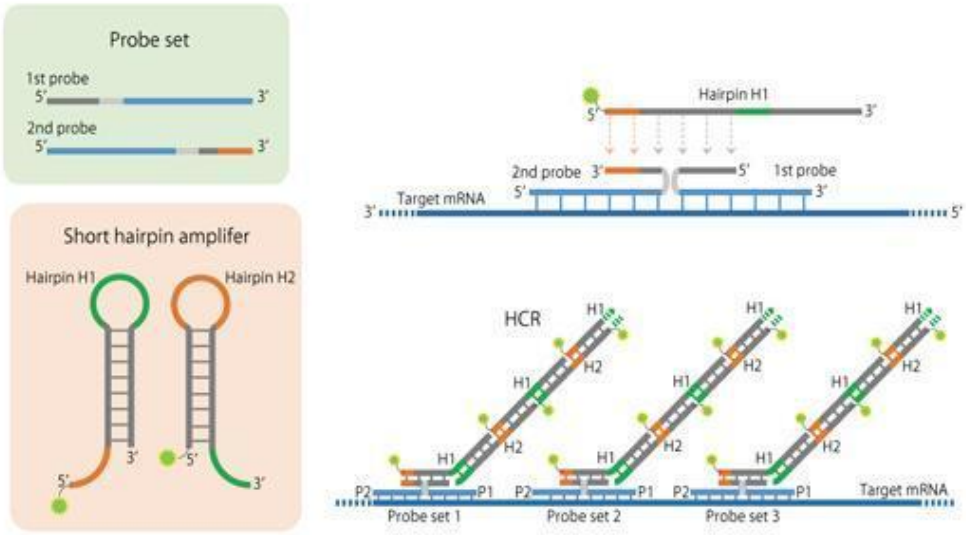
The ***in situ* hybridization chain reaction (HCR)** method utilizes the self-catalyzed elongation of nucleic acids with a hairpin structure 1) to initiate a chain polymerization reaction of labeled DNAs on tissue sections, thereby enabling visualization of target nucleic acids 2).

Because this method does not rely on antibody or enzymatic reactions, it exhibits linear signal intensity and a high signal-to-noise ratio.
The hybridization of two probes containing sequences that initiate the HCR (polymerization reaction) of the hairpin DNAs, followed by the subsequent HCR, results in fluorescently labeled DNAs specifically binding to **the target mRNA**.

HCR occurs only when the two probes hybridize in the correct positions, minimizing nonspecific reactions. The ISHpalette™ is a further shortened version of the hairpin DNA, eliminating the need for the conventional permeation treatment 3). Furthermore, because the hybridization time per molecule is reduced, the reaction reaches plateau faster, resulting in higher signal intensity.

This method is applicable to a wide range of tissues, including cultured cells, and is compatible with **paraffin sections, frozen sections, free-floating sections, adherent cells, suspension cells, and whole-mount samples**. Because staining can be performed under very mild conditions (up to 37 °C) without permeabilization treatments such as Proteinase K, it can be combined with immunostaining or used to visualize target gene expression **while preserving signals from endogenous fluorescent proteins** such as GFP. The hairpin DNAs exhibit such high specificity that even when probe concentrations are increased up to 20-fold relative to the standard protocol, no additional noise is detected. Another advantage of this method is its **high reproducibility and quantitative** performance, owing to the reduced number of procedural steps.

1) Dirks and Pierce 2004 *PNAS*
2) Choi *et al*. 2010 *Nat Biotec*; 2014 *ACS nano*; 2018 *Development*
3) Tsuneoka and Funato 2020 *Front. Mol. Neurosci*.



ISHpalette™ Short hairpin amplifier Overview

Application

Detection of mRNA in Fixed Biological Tissue Specimens

Product Lineup

Item No.	Description
IPL-G-S23	ISHpalette™ Short hairpin amplifier, SaraFluor™488-S23 (25 slides: 50µL each)
IPL-G-S45	ISHpalette™ Short hairpin amplifier, SaraFluor™488-S45 (25 slides: 50µL each)
IPL-R-S41	ISHpalette™ Short hairpin amplifier, ATTO550-S41 (25 slides: 50µL each)
IPL-R-S73	ISHpalette™ Short hairpin amplifier, ATTO550-S73 (25 slides: 50µL each)
IPL-B-C-S72	ISHpalette™ Short hairpin amplifier, Cyanine5-S72 (25 slides: 50µL each)
IPL-B-C-A161	ISHpalette™ Short hairpin amplifier, Cyanine5-A161 (25 slides: 50µL each)

Storage Conditions

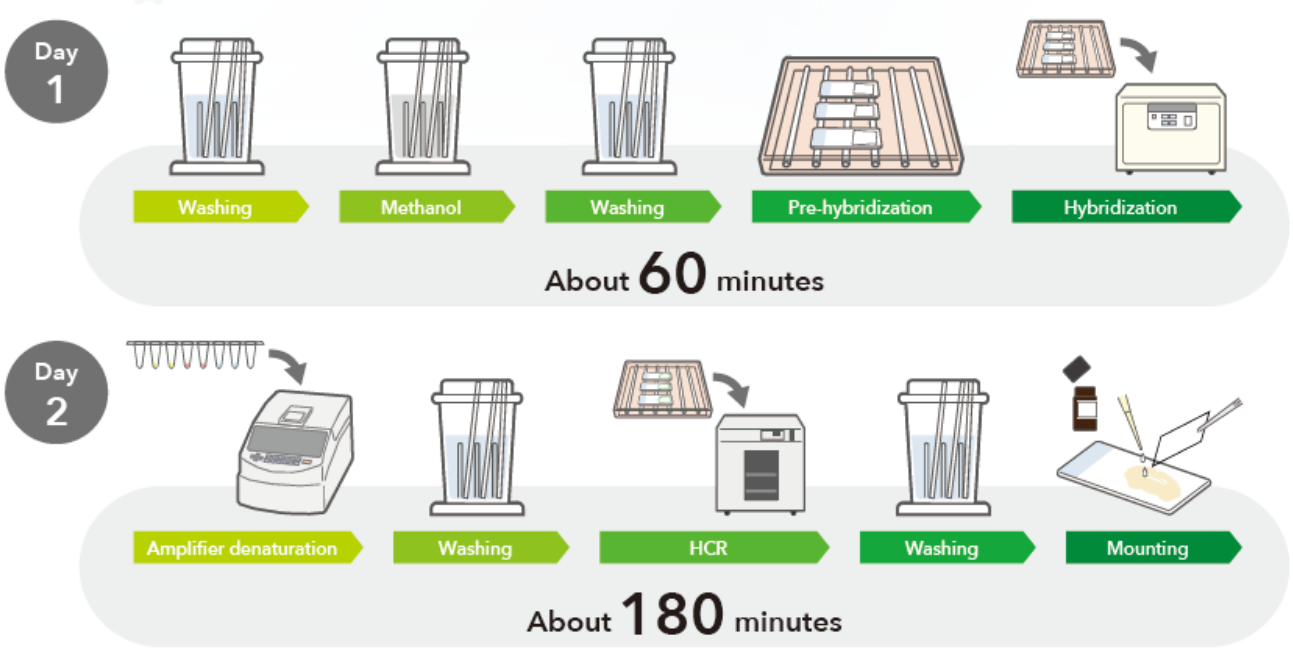
Store in a refrigerator (4–8 °C) protected from light. Freezing or storing for long periods with a small solution volume may cause fluorescent hairpin DNA to aggregate. Since the hairpin DNA is dissolved in a special buffer, do not dilute the solution during storage.

Quality Assurance

- Please use within the expiration date.
- Product quality and performance are not guaranteed if stored improperly.

This product is for research use only. It is not intended for clinical applications.

Flowchart



in situ HCR with ISHpalette™ for Frozen Sections

Detailed staining procedures are provided on the following pages. Please be sure to read them before starting the staining process.

【Day 1】

1. Permeabilization: Methanol treatment at room temperature for 10 min
2. Washing with PBST at room temperature for 5 min × 2 times
3. Pre-hybridization: Hybridization buffer treatment without target probe at room temperature–37 ℃ for 5–10 min
4. Hybridization: Hybridization buffer treatment with target probe at 37 ℃ overnight

【Day 2】

5. Heat treatment of ISHpalette™ Short hairpin amplifiers H1 and H2
6. Washing with 0.5× SSCT at 37 ℃ for 10 min × 3 times
7. Pre-amplification: Amplification buffer treatment at room temperature or 25 ℃ for ≥ 5 min
8. Amplification and detection (with nuclear staining): Treatment of Amplification buffer + ISHpalette™ Short hairpin amplifiers H1 and H2 (+ nuclear stain) at room temperature or 25 ℃ for 2 h
9. Washing with PBST at 37 ℃ for 10 min × 3 times, then with PBS for 5 min × 1 time
10. Mounting

Staining Protocol

Required Reagents

- ISHpalette™ Short hairpin amplifier H1 (store refrigerated)
- ISHpalette™ Short hairpin amplifier H2 (store refrigerated)
- Hybridization Buffer (store at –20 to –30 °C; thaw at room temperature before use)
- Amplification Buffer (store refrigerated; bring to room temperature before use)
- DNA Probe Mixture (store at -20～-30 ℃) (*see next page)
- Methanol, special grade
- PBS
- PBST（PBS, 0.1% Tween20）
- 0.5×SSCT（0.5×SSC, 0.1% Tween20）
- Mounting medium for fluorescence staining (recommended: VECTASHIELD® Vibrance™, #H-1700, VECTOR LABORATORIES)

It is generally preferable to use RNase-free water for the solutions, but this is not strictly required. In practice, the most effective way to prevent RNA degradation during the experiment is not excessive concern over contamination, but rather proceeding quickly to the hybridization step and avoiding leaving samples at room temperature or above for extended periods.

Suggested Supplies and Equipment

The experiment can be performed with equipment commonly used in molecular biology or histology laboratories. It is recommended to use sterilized or RNA-grade items such as pipette tips and staining dishes.

- Micropipettes
- 37 ℃ incubator
- Incubator with refrigeration capable of maintaining 25 ℃ (if available)
  Note: *Even if the temperature can be set to 25 ℃, incubators without a refrigeration function cannot actually maintain 25 ℃. In such incubators, the minimum achievable setting is typically at least 5 °C above room temperature.*
- Thermal cycler
- Forceps
- Parafilm
- Staining dishes
- Humidified chamber
- Coverslips
- Confocal laser scanning microscope or fluorescence microscope
  Note: *A confocal laser scanning microscope is required if detecting signals at the single-copy level.*

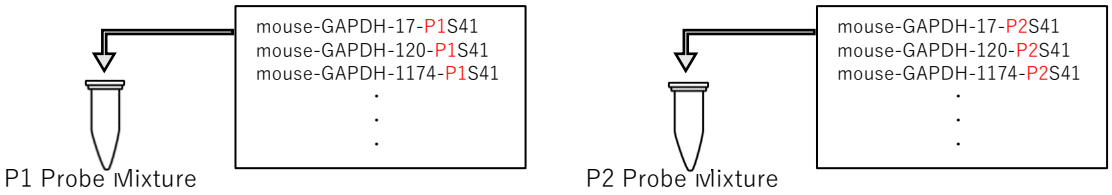
Sample Preparation

ISHpalette™ staining can be performed on standard paraffin sections, frozen sections, free-floating sections, adherent or suspension cells, and tissue blocks. To prevent RNA degradation, perform perfusion fixation with 4% PFA or immersion fixation with 10% neutral buffered formalin at 4 ℃ for at least one night. In addition, store the prepared sections or samples at –20 ℃ or below.

Preparation of Target Probe Mixture

We also offer custom design services for target probes. Please feel free to contact us for consultation.

1. For single-stranded oligo DNAs synthesized by an oligo manufacturer, dispense equal amounts of the 36-base P1 probes (5' end) and the 39-base P2 probes (3' end) into separate tubes and mix them separately.



2. Add TE buffer to each of the P1 probe mixture and P2 probe mixture to adjust the final concentration of each oligo DNA to 1–10 μM.

> Example:
> When eight types of P1 and P2 are supplied at 50 μM, 100 μL each,
> - Mix 90 μL of each P1 oligo DNA (eight types) + add 180 μL TE buffer → 5 μM P1 probe mixture
> - Mix 90 μL of each P2 oligo DNA (eight types) + add 180 μL TE buffer → 5 μM P2 probe mixture

3. Store frozen at –20 to –30 ℃ until use. Repeated freeze–thaw cycles are generally not problematic.

Staining Procedure for Frozen Tissue Sections

～ Tips Before Staining ～

- Steps 1 through 5 should be carried out quickly without delay to achieve optimal staining results. It is recommended to bring the Hybridization Buffer to room temperature in advance and prepare the probe-containing Hybridization Buffer beforehand.
- Both the Hybridization Buffer and Amplification Buffer must be fully equilibrated to room temperature before use. Because of their high viscosity, **all reagents should be thoroughly mixed using a touch mixer (3 seconds × 3 times) before use**. In addition, rather than simply dispensing the reagents onto the slide, **tilt the slide and repeatedly spread the solution across its surface to ensure even distribution**.
- Unlike conventional *in situ* hybridization methods, Proteinase K treatment, acetylation, and DNase treatment are not required. In some cases, these treatments may actually reduce the signal.

【Day 1】

1. **Preparation of Sections**
   Prepare slides with frozen tissue sections and immerse them in PBS at room temperature for 5 minutes to remove embedding compounds and other residues.

2. **Delipidation and Permeabilization: Methanol treatment at room temperature for 10 min**
   Immerse the slides in methanol at room temperature for 10 minutes to delipidize and permeabilize the cell membranes.

3. **Washing: PBST treatment at room temperature for 5 min × 2 times**
   Immerse the slides in PBST at room temperature for 5 minutes to wash. Repeat the washing step once more.

4. **Pre-hybridization: Hybridization buffer treatment at room temperature for 5 min**
   Thaw the Hybridization Buffer before use and mix thoroughly.
   Apply 100 µL of Hybridization Buffer (brought to room temperature) onto each slide to completely cover the section. Carefully place a piece of Parafilm, cut to match the section size, over the sample using forceps, taking care to avoid air bubbles. Place the slides in a humidified chamber and incubate at room temperature for 5 minutes to prevent the tissue from drying out.

5. **Preparation of Hybridization Solution Containing Target Probe (can be prepared in advance)**
   Add 2 µL of 1 µM probe mixture to 100 µL of Hybridization Buffer (final concentration approx. 20 nM). Heat-denature the prepared probe Hybridization Solution at 95 ℃ for 3 minutes, mix thoroughly, and then keep at room temperature until use.

When staining multiple genes, mix all the target probes to be used together in a single Hybridization Buffer in the same manner.

● When detecting only Gene A on two slides using SF488-S23

	Volume per slide	Final Conc.	Total（×２）
Hybridization buffer	100 µL		200 µL
Gene A-S23　P1 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Gene A-S23　P2 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Hybridization Solution Containing Probe	104 µL		208 µL

● When detecting Gene A with SF488-S23 and Gene B with AT550-S41 on two slides

	Volume per slide	Final Conc.	Total（×２）
Hybridization buffer	100 µL		200 µL
Gene A-S23　P1 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Gene A-S23　P2 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Gene B-S41　P1 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Gene B-S41　P2 Probe Mixture 1µM	2 µL	(appx. 20 nM)	4 µL
Hybridization Solution Containing Probe	108 µL		216 µL

6. Hybridization at 37 ℃ Overnight

Remove as much excess liquid from the slide as possible (completely remove liquid from areas without sections using Kimwipes or similar). Apply 100 µL of probe Hybridization Solution onto each slide to fully cover the section. Carefully place a piece of Parafilm, cut to match the section size, over the sample using forceps, making sure to avoid air bubbles. Place the slides in a humidified chamber and incubate overnight at 37 °C to prevent the tissue from drying out.

Since 0.5× SSCT at 37 °C will be used for washing the next day, place it in the incubator together to pre-warm. If the Parafilm extends beyond the slide, the Hybridization Solution may leak out, so take care to avoid any overhang.

(To be continued on Day 2.)

【Day 2】

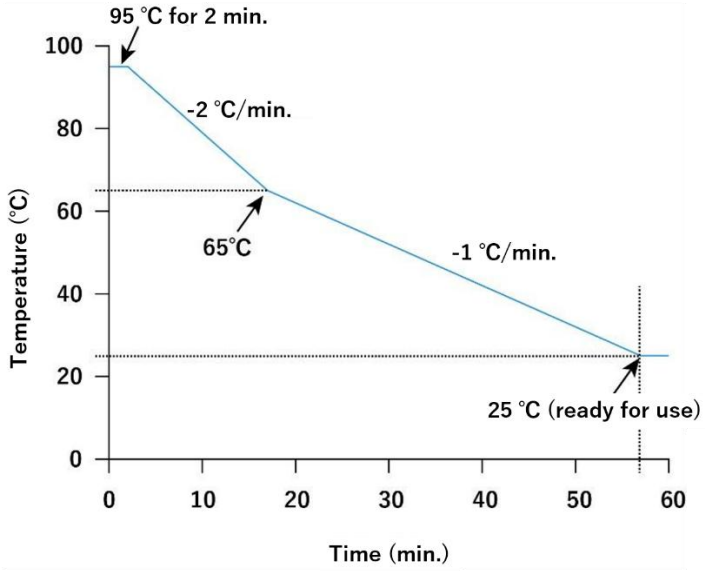
7. Heat treatment of hairpin DNA: appx. 1 hour using a thermal cycler

Calculate the required amount of ISHpalette™ Short hairpin amplifier.

(For example, if 2 µL is needed, prepare 3 µL for heat treatment to account for evaporation during heating.)

Dispense the required amounts of ISHpalette™ Short hairpin amplifier H1 and H2 into **separate PCR tubes without mixing**. Using a thermal cycler, heat at 95 ℃ for 2 minutes, then cool at a rate of 2 ℃ per minute to 65 ℃ over 15 minutes, followed by cooling at a rate of 1 ℃ per minute to 25 ℃ over 40 minutes.

Since the heat treatment is time-consuming, proceed with steps 8, 9, and 10 during this process.



8. Preparation of Amplification Buffer

Remove the Amplification Buffer from the refrigerator and leave it at room temperature for at least 30 minutes. During this time, proceed with steps 9 and 10.

Note: Depending on the room temperature, the buffer may appear cloudy, which is due to micelle formation and is not a problem. ***The buffer may also separate, so after standing at room temperature for 30 minutes, be sure to mix thoroughly before use.***

9. Washing with 0.5× SSCT at 37 ℃ for 10 min × 3 times

Carefully remove the Parafilm using forceps, then immerse the slides in pre-warmed 0.5× SSCT at 37 °C for 10 minutes to wash. Repeat the washing step two more times.

10. Pre-amplification with Amplification Buffer at 25 ℃ for ≥ 5 min

Outline the tissue with a Pap pen, then apply Amplification Buffer (brought to room temperature) onto the slide. Place the slide in a humidified chamber and incubate at room temperature or 25 ℃ for 5 minutes, ensuring the tissue does not dry out.

11. Preparation of Detection Solution

For each slide, mix 2 µL each of ISHpalette™ Short hairpin amplifiers H1 and H2 with 100 µL of Amplification Buffer, then vortex for 3 seconds × 3 times.

When high concentrations of hairpin DNA come into contact with each other, they may cause background noise; therefore, change pipette tips when mixing.
If nuclear staining is required, add the stain at this step (for Hoechst, adjust to a final concentration of 1 µg/mL).
For multicolor staining, mix all hairpin amplifiers H1 and H2 for each color together in a single Amplification Buffer in the same manner (see below).

- When detecting only Gene A on two slides using SF488-S23

	Volume per slide	Total（×２）
Amplification buffer	100 µL	200 µL
SF488-S23　H1	2 µL	4 µL
SF488-S23　H2	2 µL	4 µL
Detection Solution	104 µL	208 µL

- When detecting Gene A with SF488-S23 and Gene B with AT550-S41 on two slides, while simultaneously performing nuclear staining

	Volume per slide	Total（×２）
Amplification buffer	100 µL	200 µL
SF488-S23　H1	2 µL	4 µL
SF488-S23　H2	2 µL	4 µL
AT550-S41　H1	2 µL	4 µL
AT550-S41　H2	2 µL	4 µL
Hoechst (100µg/mL)	1 µL	2 µL
Detection Solution	109 µL	218 µL

12. Amplification: Amplification Buffer containing Short hairpin amplifiers at 25 ℃ within 2 hours

Remove as much excess liquid from the slide as possible (completely remove liquid from areas without sections using Kimwipes or similar). Immediately after mixing, apply the Detection Solution prepared in step 11 onto the sections as quickly as possible. After application, **tilt the slide to spread and mix the Detection Solution evenly over the section.** Place the slides in a humidified chamber and incubate at 25 ℃ or room temperature for 2 hours, ensuring the tissue does not dry out.

The reaction time must be within 2 hours from the start of mixing the hairpin DNA in step 11. A key to successful experiments is to maintain a sufficient volume of Detection Solution without dilution. **Tilting the slide at least once every 30 minutes to mix the Detection Solution on the slide** helps achieve uniform signal development.

13. **Washing with PBST at 37 ℃ for 10 min × 3 times; with PBS at room temperature for ≥ 5 min**
    Immerse the slides in PBST at 37 °C for 10 minutes to wash. Repeat this washing step two more times.
    Then immerse the slides in PBS at room temperature for 5 minutes to wash.

14. **Mounting**
    Mount the sections using an anti-fade mounting medium.
    After mounting, store at 4 ℃ until observation and imaging. (Depending on the mounting medium, observation is generally possible for about one week without issues.)

Procedure for Combined Use with Immunostaining

Perform staining according to the ISHpalette™ Short hairpin amplifier protocol.
↓
After the washing in step 13, proceed with blocking and then perform immunostaining.

Procedure for Paraffin Sections

In the pretreatment in step 1, perform deparaffinization, de-xylene treatment, and hydration, then immerse the sections in PBS. After that, proceed with staining from step 2. Activation treatment or high-temperature treatment is not required.

Procedure for Whole-Mount Samples

In the pretreatment in step 1, perform 75% MeOH/PBST for 5 min → 50% MeOH/PBST for 5 min → 25% MeOH/PBST for 5 min, then proceed with staining from step 3.
For hybridization and amplification, place one sample per well in a 96-well plate and react in 100 µL of solution, which allows the experiment to be performed with a small volume of reagents.
For nuclear staining, it is recommended to perform it simultaneously during step 9, as this facilitates deeper penetration.
After completing up to step 13, clear the samples using SeeDB for observation. (*Note: Clearing reagents such as Scale or CUBIC often contain urea, which is not compatible with HCR products and may cause signal loss. If using such reagents, post-staining fixation may allow their use, but please be aware that HCR products may degrade.*)
For detailed information on the SeeDB method, please refer to the website SeeDB Resources by Dr. Takeshi Imai and colleagues: https://sites.google.com/site/seedbresources/
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Key Points for Section Observation

- A single copy of mRNA is detected as a very small, punctate signal (it becomes harder to distinguish when expression levels are high).
- Use a confocal microscope to detect signals at the single-copy level.
- For single-copy detection, start with an objective lens of NA 0.7 or higher, a relatively long exposure time (≥ 6 µs/pixel per wavelength is recommended, but use a slower scanning speed for higher-

resolution imaging), and strong laser power. Averaging has little effect, so a minimal number of scans is sufficient.

- If washing is insufficient, granular background signals may appear across the sample. In such cases, try extending the washing step before mounting.
- Always confirm with a negative control or unstained section that autofluorescence is not excessively strong.
- If autofluorescence is strong, this can sometimes be resolved by using an oil-immersion lens or by preparing thinner sections.
- If signals are still not visible even after considering the above, it is unlikely but possible that the probe sequence is not optimal. In such cases, try increasing or modifying the probe binding region against the mRNA.

- This protocol is subject to change without notice for product improvements, specification updates, or enhancements.
- This product does not cover or guarantee the performance or use of other companies' products.
- This development product has been co-developed and commercialized in collaboration with Toho University, utilizing the technology (Patent No. 7482506) developed by Associate Professor Yousuke Tsuneoka, Department of Anatomy (Division of Microscopic and Morphological Anatomy), Faculty of Medicine, Toho University.