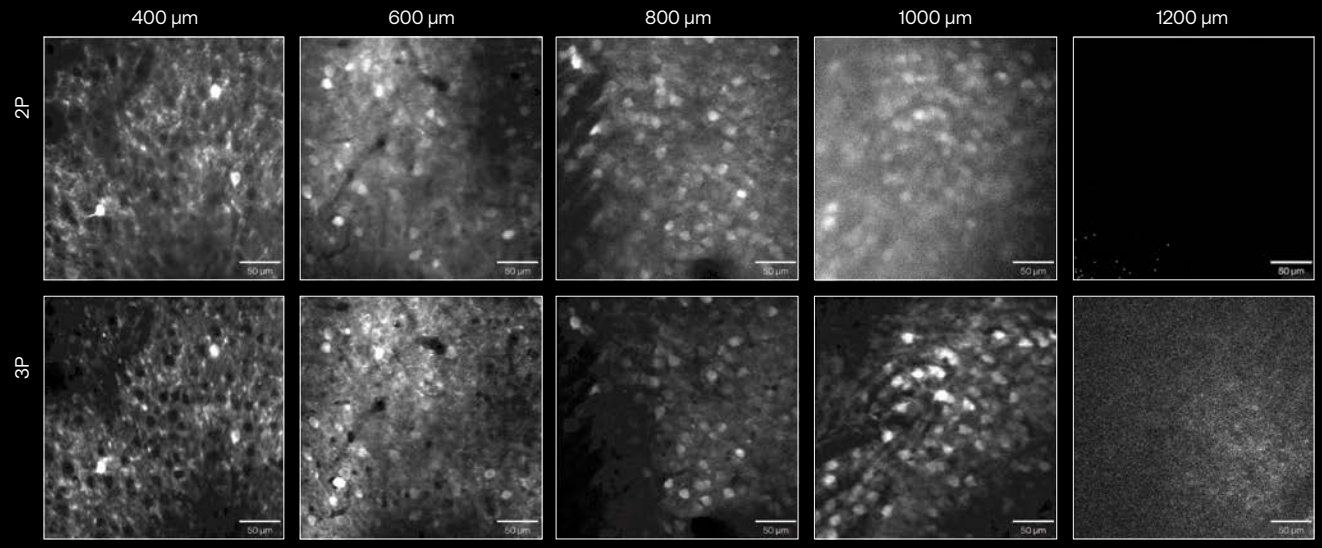
# Nonlinear Microscopy

# In vivo three-photon imaging

Recording real-time single-neuron activity in deep brain layers of awake animals is crucial for understanding behavior, brain connectivity, and function. These applications have been advanced by neuron imaging and stimulation using high-power, high-pulse-energy, medium-repetition-rate lasers tunable in the SWIR range, which includes the biological transparency windows at 1.3 µm and 1.7 µm. Three-photon microscopy has been shown to provide higher image contrast at greater depths. **CRONUS-2P, CRONUS-3P,** and **ORPHEUS** OPA are state-of-the-art choices for two- and three-photon-excited fluorescence (2PEF, 3PEF) and harmonic-generation (SHG, THG) imaging in deep tissues.

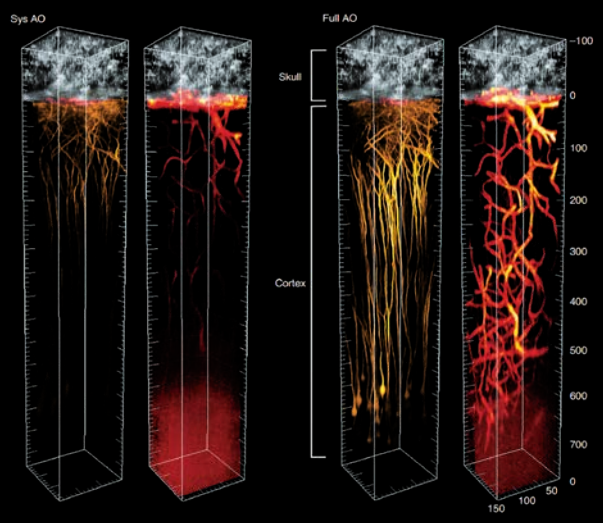
## 2P and 3P calcium imaging at depth in the mouse brain




Comparison of in vivo 2P and 3P calcium imaging of mouse visual cortex GCaMP neurons on a Thorlabs Bergamo II microscope using a typical 2P laser and Light Conversion's **CRONUS-3P** laser at 920 nm and 1300 nm, respectively.

Courtesy of CSHL ISFNS 2024 school organizers, Willis Broden Jr. and Sergey Matveev (Thorlabs).

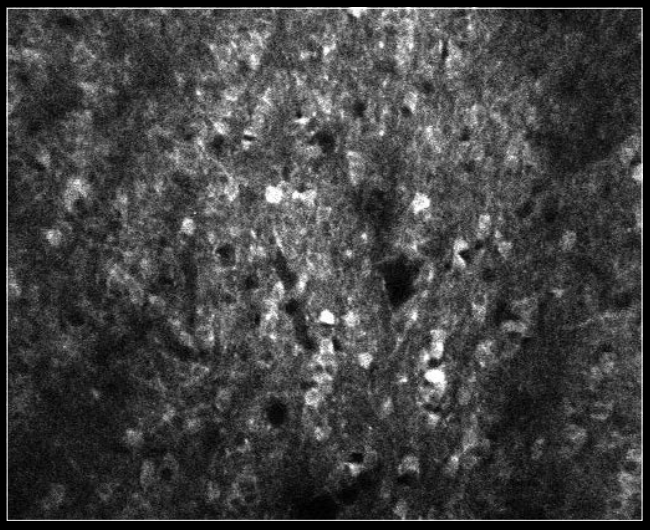
## 3P AO brain imaging through the intact skull




**ORPHEUS-F** excitation at 1300 nm enabled imaging up to 1.1 mm below the pia within the intact brain.

Courtesy of Jianan Y. Qu group, the Hong Kong University of Science and Technology. Source: Zh. Qin et al., Deep tissue multi-photon imaging using adaptive optics with direct focus sensing and shaping, Nature Biotechnology 40 (2022).

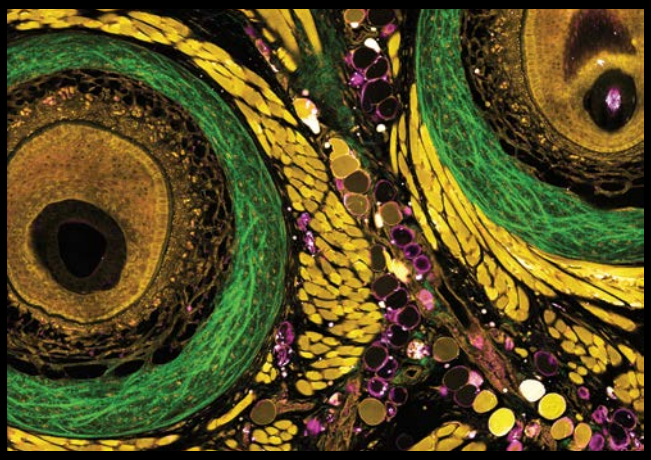
## 3P anatomical imaging of the mouse olfactory bulb



Mouse olfactory bulb with inhibitory cells labelled with GCaMP8 s. Anatomical Z-stack imaged in 3P at 620 µm.

Courtesy of Fred Marbach, Andreas Schaefer lab, The Francis Crick Institute.

# Label-Free In Vivo Imaging

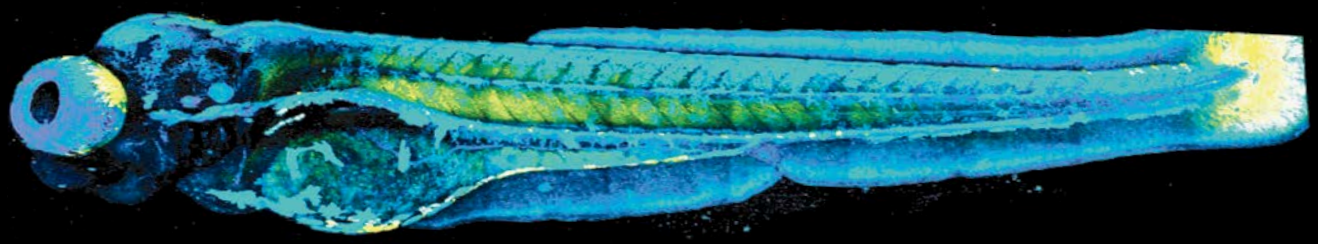


Understanding biological complexity requires minimally invasive imaging tools capable of providing multiplexed molecular contrasts. To address this need, S. You's laboratory at the Massachusetts Institute of Technology is developing a non-invasive, label-free microscopy approach using **CRONUS-3P** to visualize biosystems.

As part of a study on neuropathic pain, the image reveals the rich microenvironment of an unprocessed, intact mouse whisker pad: collagen capsule (green), comprising the follicle with muscles (yellow) supporting it, adipocytes (purple), stromal cells, and immune cells.

Courtesy of Sixian You group, Massachusetts Institute of Technology.

## Multimodal 3D in vivo imaging of zebrafish



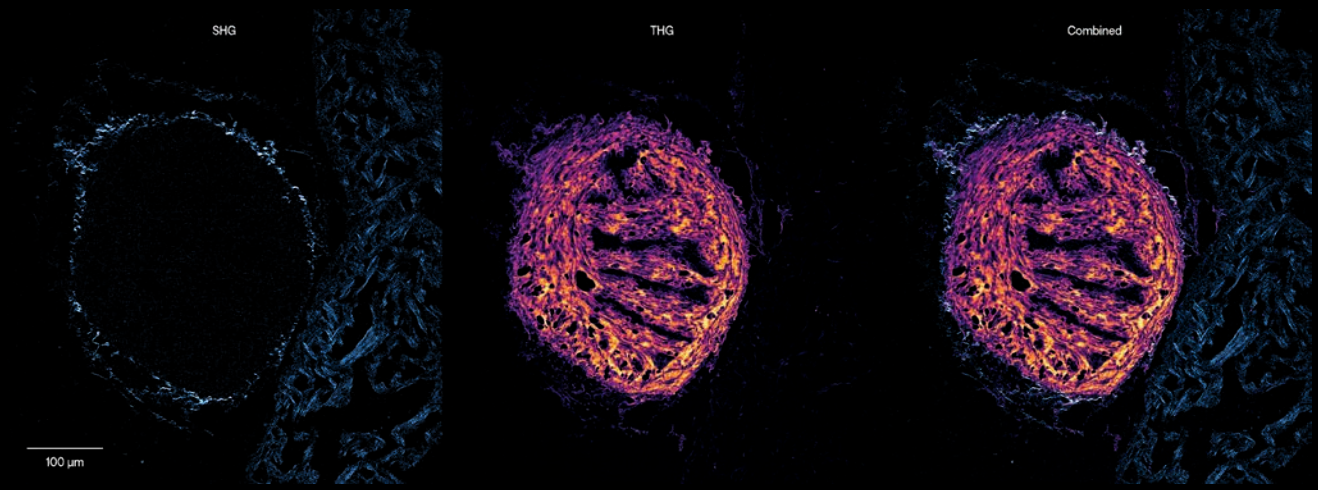
Multimodal 3D label-free imaging of a live 4dpf zebrafish embryo. The embryo was healthy after imaging.
3PF: green, SHG transmission: yellow, THG epi: dark blue, THG transmission: cyan.
Excitation: 1300 nm, XY pixel size: 0.39 µm/px, Z stack: 522 µm range, 1 µm step, Mosaic: 11 x 2 of 400 x 400 µm tiles, Total imaging time: 12 h.

Courtesy of Luigi Bonacina group, University of Geneva.

## Combined SHG and THG imaging

Adult zebrafish heart ventricle section from a scar formation study. The brightfield image is stained with Masson's trichrome (MT), connective tissue is blue, muscle is red/brown.

SHG and THG images reveal collagen and muscle structure at the periphery of bulbus arteriosus, while MT-stained elastin is visualized in the center in THG.

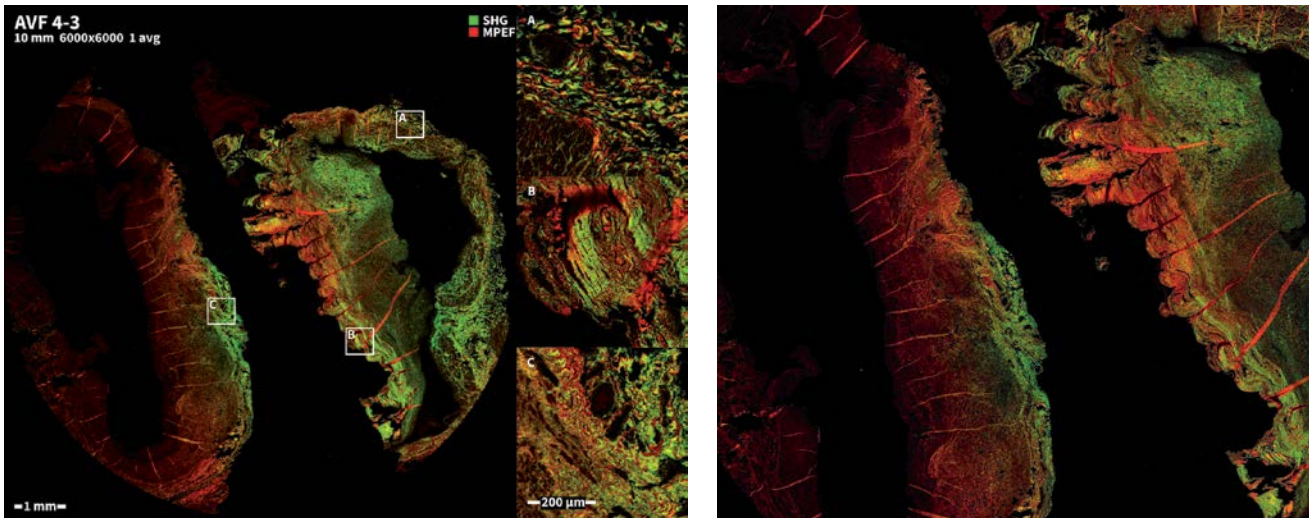



Adult zebrafish heart ventricle section imaged using the **FLINT** femtosecond oscillator.

Samples courtesy of Justas Lazutka, Vilnius University Life Sciences Center.
Imaging courtesy of Virginijus Barzda group, Vilnius University Faculty of Physics.

## Multimodal nonlinear microscopy

Multiphoton microscopy image of two transverse sections of a human vein exhibiting a fibrotic intima, acquired with a large-field-of-view microscope with a **FLINT** oscillator. The image was obtained using 1030 nm excitation in a single scan covering a 10 mm diameter field of view with a 0.8 NA objective. Collagen is visualized via second-harmonic generation (SHG) and displayed in green, while multiphoton-excited fluorescence (MPEF) from Verhoeff-stained elastic fibers is shown in red. A reduced-resolution 1000 × 1000 pixel overview thumbnail is presented on the left. Fullresolution regions of interest (ROIs) corresponding to the areas marked by white squares in the overview image are displayed on the right.

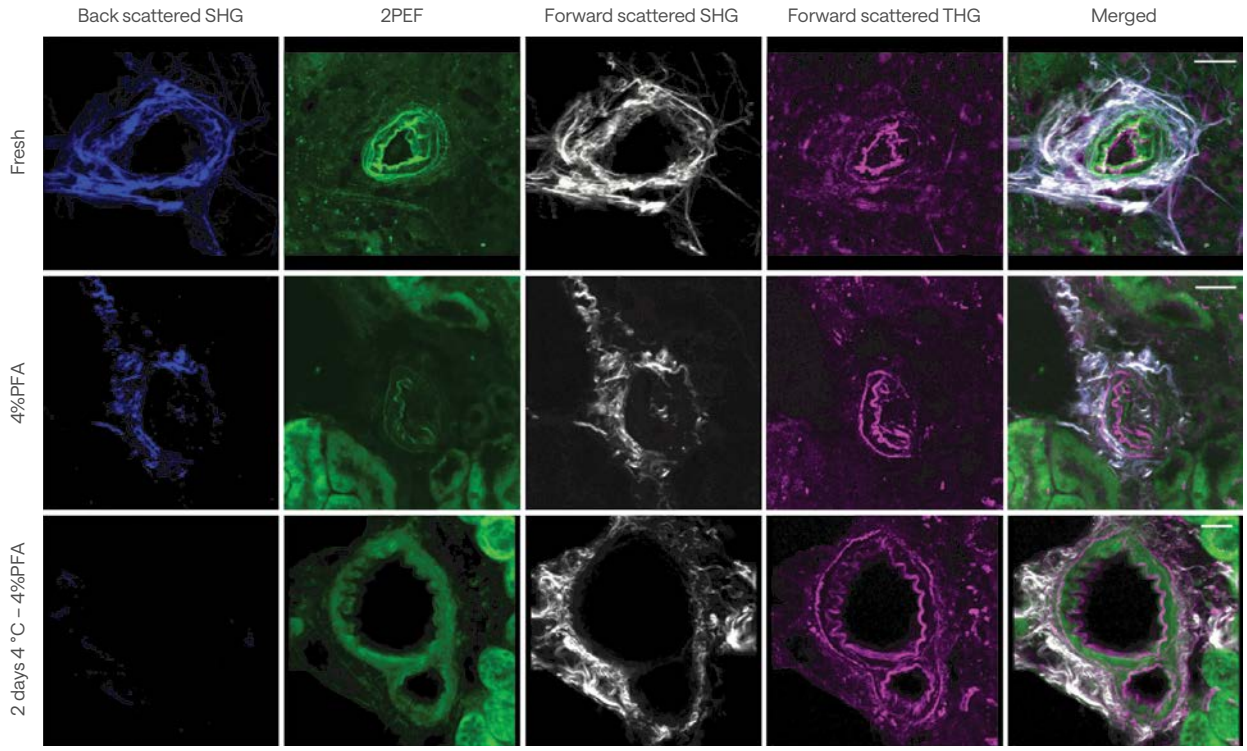



Human arteriovenous fistula, two transverse thin sections.

Courtesy of Virginijus Barzda group, Vilnius University.

## SHG, THG, and 2P imaging

Fixation methods, such as formalin, are commonly used to preserve tissue and keep its structure as close as possible to the native condition. However, fixatives chemically interact with tissue molecules and may modify their structure. Taking advantage of the SHG and THG emission capabilities of such components, nonlinear 2P microscopy and the **CRONUS-2P** femtosecond laser were used to evaluate the effect that preservation methods, such as chemical fixatives, have on the nonlinear capabilities of protein components within mouse tissues.



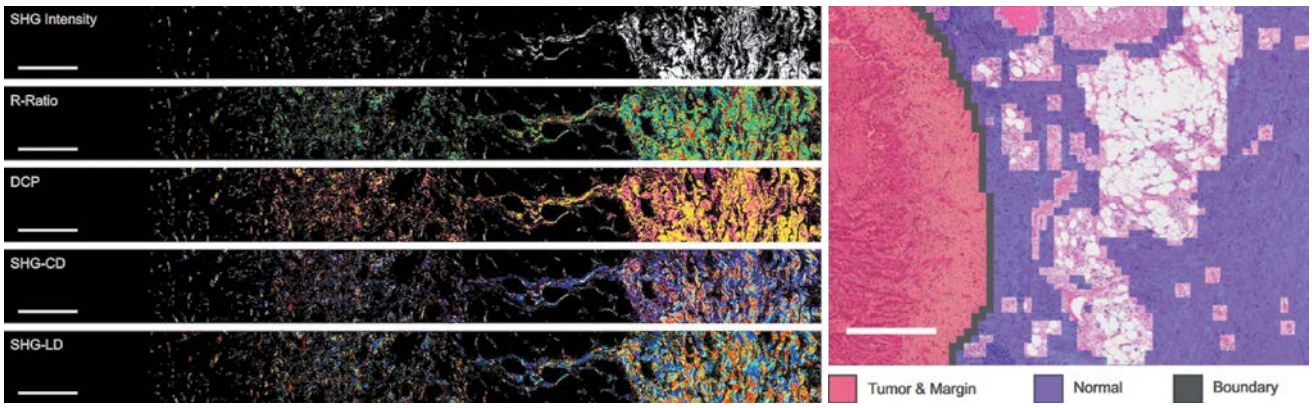

SHG signals from collagen, 2PEF and THG signals from elastin in vibratome sections of mouse kidney after different treatments, acquired using the **CRONUS-2P** femtosecond laser source.

Courtesy of Frauke Alves and Fernanda Ramos-Gomes, Max-Planck Institute for Multidisciplinary Sciences, Germany.

## Widefield polarimetric SHG microscopy

Cancer diagnosis and surgical treatment rely on imaging techniques that demand specificity and high throughput. Polarization-resolved second-harmonic generation (P-SHG) microscopy shows potential for visualizing structural changes in collagen networks and the extracellular matrix associated with tumor development. Moreover, P-SHG imaging is label-free and compatible with live tissue imaging at depth. However, traditional raster scanning methods are too slow for clinical applications, and interpreting the structural sensitivity of P-SHG can be challenging.

Nonlinear widefield microscopy addresses these limitations by utilizing amplified femtosecond lasers to increase imaging throughput and field of view. Additionally, machine learning (ML) techniques enable data-driven analysis, facilitating tasks such as automating tumor margin delineation and scoring. By leveraging **CARBIDE** and **PHAROS** lasers in conjunction with ML-augmented widefield microscopy, we can potentially extend the benefits of nonlinear microscopy to the scale required for biomedical and clinical applications.



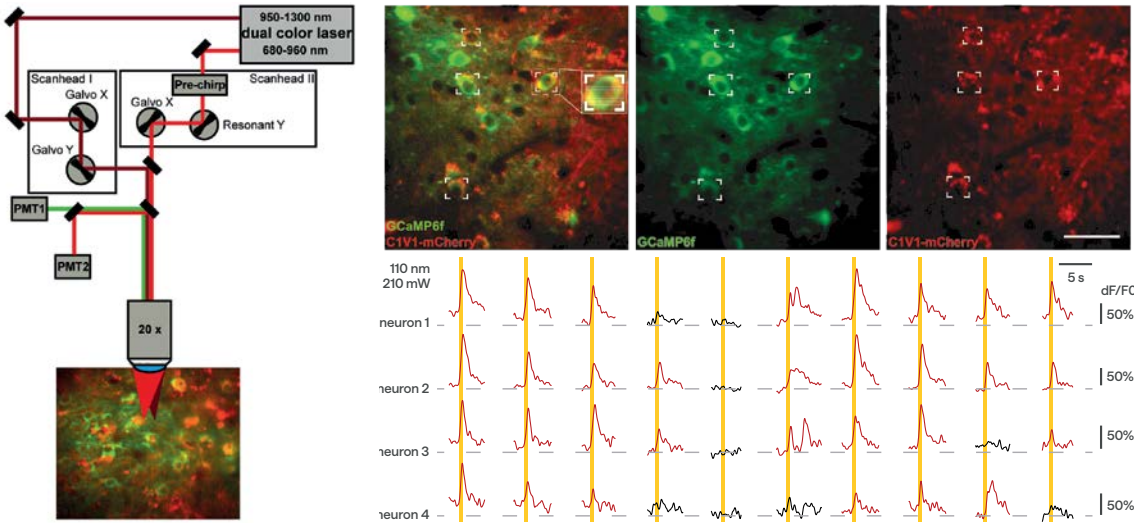

Large-area widefield P-SHG microscopy of human lung tissue tumor margins conducted using the **PHAROS** laser. Image parameters, including SHG intensity, R-ratio, and degree of circular polarization, as well as SHG circular and linear dichroism, are employed in unsupervised ML algorithms to determine the tumor boundary.

Courtesy of Virginijus Barzda group, University of Toronto, and Brian C. Wilson group, Princess Margaret Cancer Centre. Source: Mirsanaye et al., Unsupervised determination of lung tumor margin with widefield polarimetric second-harmonic generation microscopy, Scientific Reports 12 (2022).

## All-optical physiology

Despite the ability of 3-photon imaging to provide high-contrast within deep tissue, many experimental questions are still better addressed with 2-photon imaging – particularly when video-rate acquisition is required. For these applications, the **CRONUS-2P** laser offers the ultimate solution, featuring three optically synchronized outputs, two of which are independently tunable. This three-beam source enables a wide range of multiphoton excitation pathways, many of which are inaccessible with traditional single- or dual-beam solutions.

For experiments that require simultaneous activation of large numbers of cells, the **CARBIDE** laser remains a proven, high-performance choice for demanding two-photon optogenetics.




2P optogenetic stimulation of individual neurons using **CRONUS-2P**.

Courtesy of Albrecht Stroh group, University Medical Center Mainz and Leibniz Institute for Resilience Research. Source: T. Fu et al., Exploring two-photon optogenetics beyond 1100 nm for specific and effective all-optical physiology, iScience 24 (2021).

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