Application Note

Cell Analysis

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Agilent NovoCyte Flow Cytometer with 561 nm Laser

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Introduction

The expansion of flow cytometry applications from cellular analysis to molecular and genomic analysis, and the increase in availability of monoclonal antibodies and fluorochromes have heightened the demand for flow cytometers with expanded fluorescence detection channels. Agilent NovoCyte 3000 VYB (405 nm, 561 nm, 488 nm) and NovoCyte 3000 RYB (640 nm, 561 nm, 488 nm) flow cytometers, configured with a 561 nm laser, are developed to meet the needs of multicolor analysis, broadening application capabilities in basic research and clinical studies.

The advantages of using 561 nm laser-configured NovoCyte 3000 VYB and NovoCyte 3000 RYB flow cytometers include:

- Enhancing excitation of phycoerythrin (PE)-conjugated antibodies with 561 nm excitation and increasing detection sensitivity for dim signals.
- Eliminating or minimizing Fluorescein isothiocyanate (FITC) to PE spillover by independent excitation with 488 and 561 nm lasers.
- Favoring the detection of multiple fluorescent proteins, such as green fluorescent protein (GFP), DsRed, and mCherry; fulfilling multicolor analysis of various applications.

Optical configurations of a NovoCyte flow cytometer

Optical configurations of NovoCyte 3000 VYB, NovoCyte 3000 RYB, and NovoCyte 3000 are listed in Table 1. All the data in this application note for 561 nm laser excitation were obtained from the NovoCyte 3000 VYB or NovoCyte 3000 RYB flow cytometers. Data for 488 nm laser excitation was from a NovoCyte 3000 flow cytometer.

High sensitivity detection of PE fluorochromes

The PE series of fluorochromes (PE, PE-Texas Red, PE-CF594, PE-Cy5, PE-Cy5.5, and PE-Cy7) have a primary absorption peak of 565 nm, with secondary absorption peaks at 496 and 545 nm that can be excited by either 488 or 561 nm lasers. However, the higher excitation efficiency of a 561 nm laser results in brighter fluorescence signals and detection sensitivity, manifested in improved stain index and separation of positive and negative populations (Figure 1).

Table 1. Optical configurations of the 13 to 14-color Agilent NovoCyte 3000 flow cytometers, including excitation lasers, standard filter sets for detection, and representative fluorochromes or fluorescent proteins for each channel.

Agilent NovoCyte 3000 VYB			
Laser	Channel	Filter (nm)	Fluorochrome/ Fluorescent Protein
405 nm	VL1	445/45	DAPI, Pacific Blue, Brilliant Violet 421, Alexa Fluor 405
	VL2	530/30	AmCyan, Alexa Fluor 430, Brilliant Violet 510
	VL3	586/20	Pacific Orange, Brilliant Violet 570
	VL4	615/20	Qdot 605, Brilliant Violet 605
	VL5	660/20	Qdot 655, Brilliant Violet 650
	VL6	780/60	Qdot 800, Brilliant Violet 786
488 nm	BL1	530/30	FITC, Alexa Fluor 488, GFP, YFP
	BL2	586/20	EYFP
	BL3	615/20	PI
	BL4	660/20	PerCP, 7-AAD
561 nm	YL1	586/20	PE, PI, DsRed, tdTomato
	YL2	615/20	PE-Texas Red/ECD, PE-Dazzle 594, mCherry
	YL3	660/20	PE-Cy5, APC, 7-AAD, mPlum
	YL4	780/60	PE-Cy7, PE-Alexa Fluor 750

Agilent NovoCyte 3000 RYB			
Laser	Channel	Filter (nm)	Fluorochrome/ Fluorescent Protein
488 nm	BL1	530/30	FITC, Alexa Fluor 488, GFP, YFP
	BL2	586/20	PI, YFP
	BL3	615/20	PI
	BL4	660/20	PerCP, 7-AAD
	BL6	695/40	PerCP-Cy5.5, PerCP-eFluor 710
561 nm	YL1	586/20	PE, PI, DsRed, tdTomato
	YL2	615/20	PE-Texas Red/ECD, PE-DazzleTM 594, mCherry
	YL3	660/20	PE-Cy5, APC, 7-AAD, mPlum
	YL4	695/40	PE-Cy5.5, PE-Alexa Fluor 680
	YL5	780/60	PE-Cy7, PE-Alexa Fluor 750
640 nm	RL1	660/20	APC, Alexa Fluor 647
	RL2	695/40	Alexa Fluor 700, APC-Alexa Fluor 700
	RL3	780/60	APC-Cy7, APC-H7, APC-Alexa Fluor 750

Agilent NovoCyte 3000 VBR			
Laser	Channel	Filter (nm)	Fluorochrome/ Fluorescent Protein
405 nm	VL1	445/45	DAPI, Pacific Blue, Brilliant Violet 421, Alexa Fluor 405
	VL2	530/30	AmCyan, Alexa Fluor 430, Brilliant Violet 510
	VL3	572/28	Pacific Orange, Brilliant Violet 570
	VL4	615/20	Qdot 605, Brilliant Violet 605
	VL5	675/30	Qdot 655, Brilliant Violet 650
	VL6	780/60	Qdot 800, Brilliant Violet 786
488 nm	BL1	530/30	FITC, Alexa Fluor 488, GFP, YFP
	BL2	572/28	PE, PI
	BL3	615/20	PI, PE-Texas Red/ECD, PE-Dazzle 594
	BL4	675/30	PerCP, PE-Cy5, 7-AAD
	BL5	780/60	PE-Cy7, PE-Alexa Fluor 750
640 nm	RL1	675/30	APC, Alexa Fluor 647
	RL2	780/60	APC-Cy7, APC-H7, APC-Alexa Fluor 750

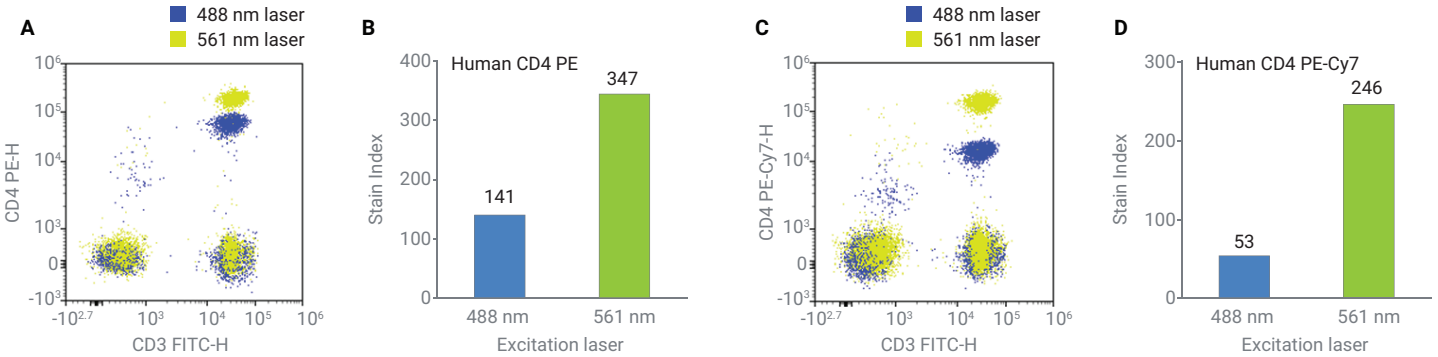

Figure 1. Human peripheral blood was stained with two different markers and analyzed using either excitation from the 488 or 561 nm lasers. (A) CD3 FITC/CD4 PE staining, (B) stain index was calculated using either 488 or 561 nm excitation. (C) CD3 FITC/CD4 PE-Cy7 staining. (D) Stain index was calculated using either 488 or 561 nm laser excitation.

Stain index is calculated by the following formula:

$$\text{Stain index} = \frac{(MFI_{Positive} - MFI_{Negative})}{(2 \times SD_{Negative})}$$

MFI = Mean fluorescence intensity
SD = Standard deviation
Positive = Positive population
Negative = Negative population

Elimination of FITC (or GFP) to PE spillover

FITC (or GFP) and PE are two commonly used fluorochromes that have overlapping emission spectra when excited by a 488 nm laser. When this occurs, compensation is generally applied to eliminate the spillover of the FITC signal into the PE detector. Using NovoCyte 3000 VYB (405 nm, 488 nm, 561 nm) and NovoCyte 3000 RYB (488 nm, 561 nm, 640 nm) flow cytometers, FITC (or GFP) and PE can be excited independently by 488 nm and 561 nm lasers, eliminating the spillover of FITC to PE and achieving better resolution of the PE signal for identifying populations with dim signals (Figure 2).

Detection of fluorescent proteins

Fluorescent proteins are useful tools for examining target protein expression and cell transfection efficiency. A 561 nm laser allows for the detection of most commonly used fluorescent proteins, such as GFP, mCherry, and DsRed, with high efficiency and sensitivity (Figure 3). Since 561 nm is closer than 488 nm to the maximum excitation wavelength for mCherry and DsRed, a higher excitation efficiency is achieved by the 561 nm laser.

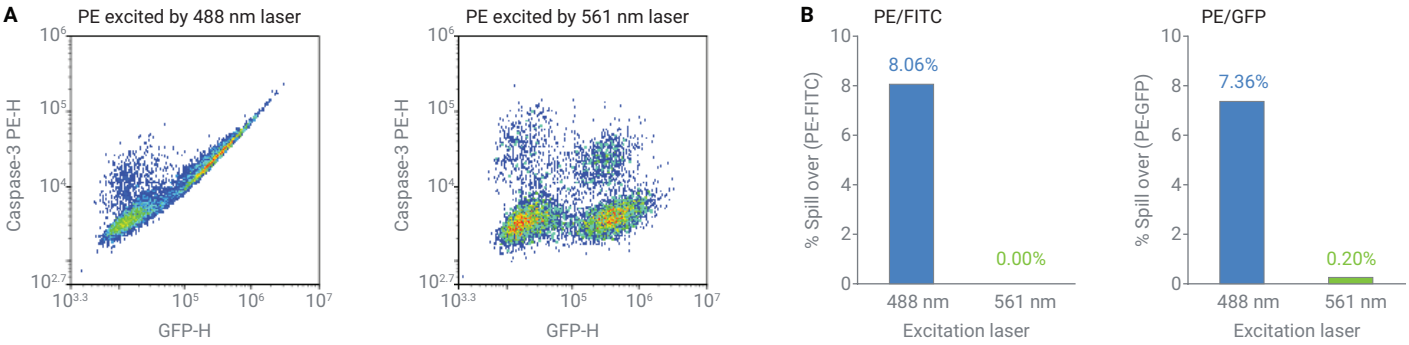

Figure 2. Independent excitation of FITC (or GFP) and PE by 488 nm and 561 nm lasers eliminates the spillover from the FITC signal to the PE detector. (A) MG132 treated A549-GFP cells were permeabilized, stained with PE for active caspase-3 (left: both GFP and PE were excited with 488 nm laser; right: GFP and PE were excited by 488 nm and 561 nm lasers, respectively). (B) Calculated %spillover of FITC into the PE detector by simultaneous excitation with 488 nm laser (left) or independent excitation with 488 nm laser and 561 nm lasers (right).

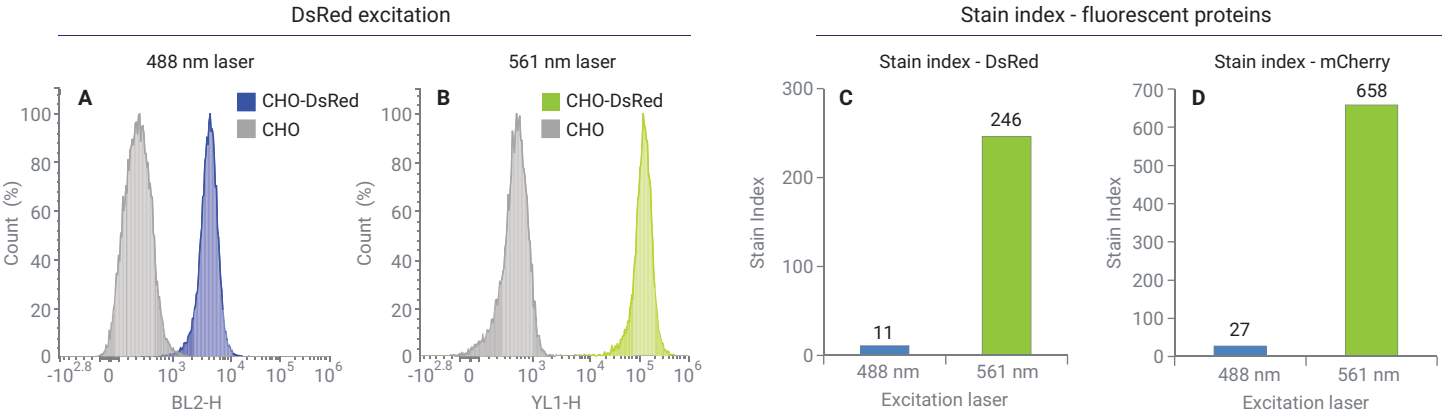

Figure 3. (A,B) Parental CHO cells and CHO cells stably expressing DsRed were detected by excitation with the 488 or 561 nm laser. (C) Excitation of DsRed with the 561 nm laser increased the stain index to 22.4-fold of that by the 488 nm laser, while the (D) excitation of mCherry with 561 nm laser increased the stain index 24.3-fold of that by the 488 nm laser.

Fulfilling multicolor analysis in various applications using a 561 nm laser

NovoCyte 3000 VYB and NovoCyte 3000 RYB flow cytometers configured with a 561 nm laser fulfill multicolor analysis of various applications. A four-color lymphocyte subtyping panel (CD3 FITC/CD8PE/CD45 PerCP/CD4 APC and CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC) can be carried out on a NovoCyte 3000 RYB or NovoCyte 3000 VYB with YL3 channel for APC detection (Figure 4). Similar analyses can readily be established on both NovoCyte 3000 RYB and 6-color (Figure 5) and 8-color (Figure 6) panels for lymphocyte subtyping.

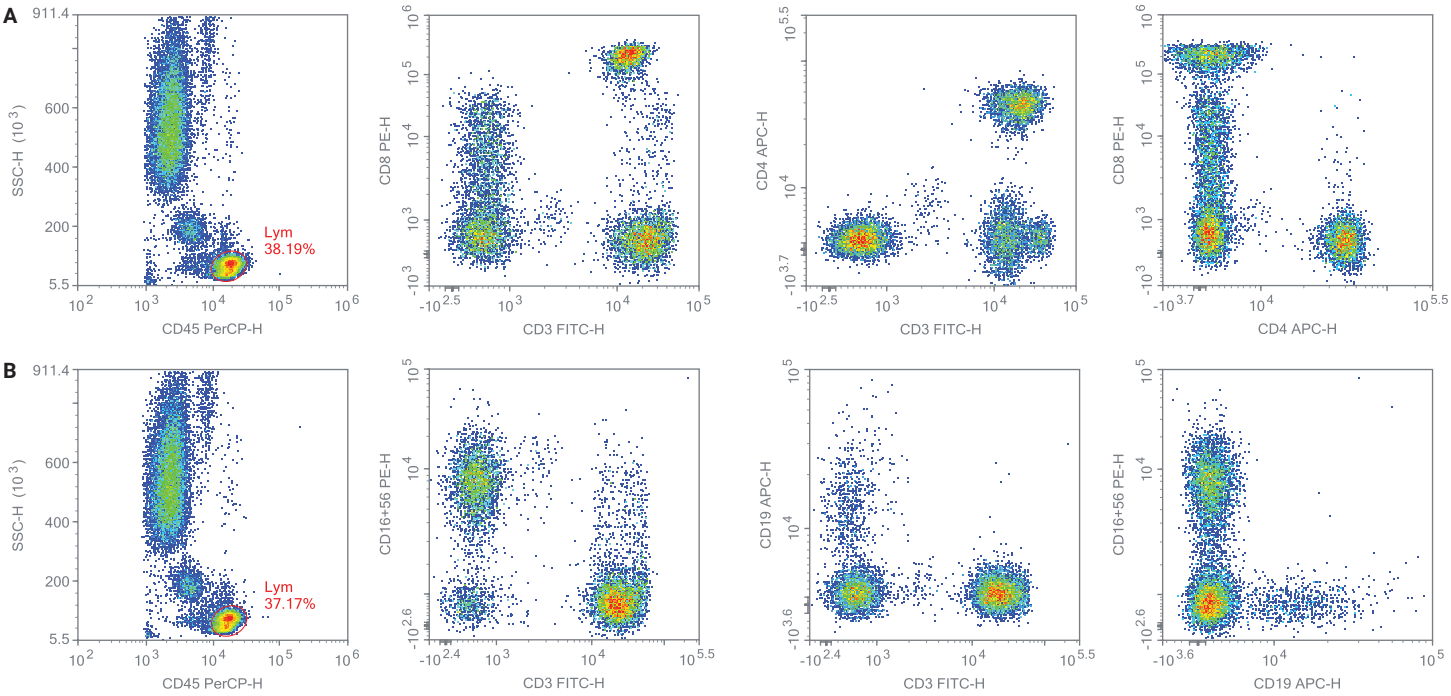

Figure 4. Lymphocyte subtyping of human peripheral blood on the Agilent NovoCyte 3000 VYB. (A) Lymphocyte subtyping using a four-color panel of CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC; (B) Lymphocyte subtyping using a four-color panel of CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC. PE and APC were detected through excitation using the 561 nm laser.

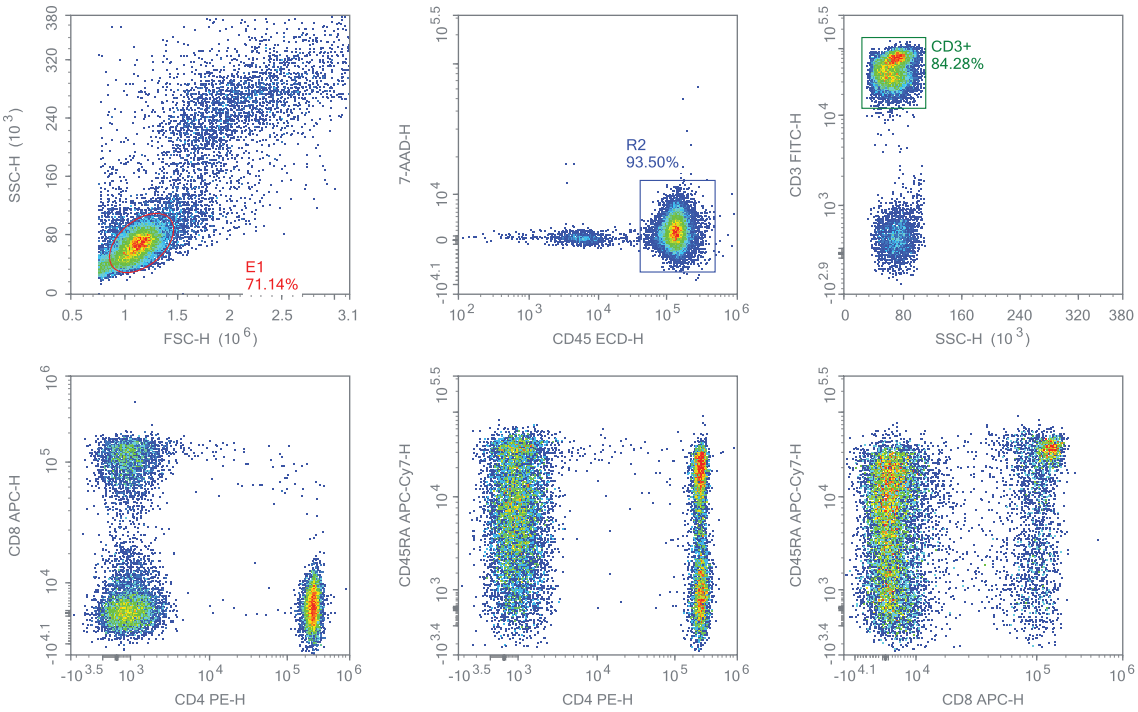

Figure 5. Lymphocyte subtyping of human PBMCs using a six-color panel of CD3 FITC/CD4 PE/CD45 ECD/CD8 APC/CD45RA APC-Cy7 on an Agilent NovoCyte 3000 VYB. Dead cells were excluded by 7-AAD staining. PE, APC, and APC-Cy7 were detected through excitation using the 561 nm laser.

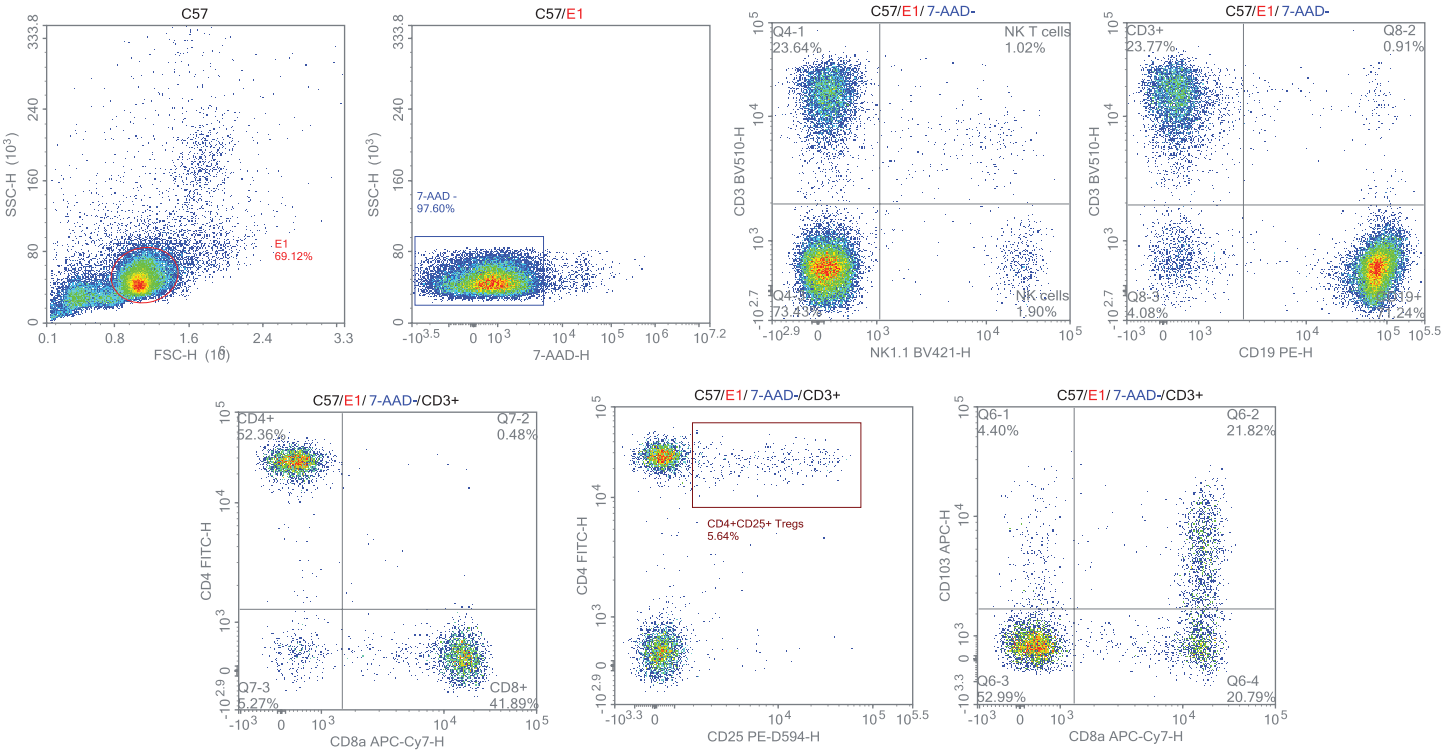

Figure 6. Freshly isolated C57 BL/B6 mouse spleen cells were stained with CD4 FITC/CD19 PE/CD25 PE-Dazzle 594/CD103 APC/CD8a APC-Cy7/NK-1.1 BV421/CD3 BV510, and lymphocyte subtyping was analyzed using an Agilent NovoCyte 3000 VYB. Dead cells were excluded by 7-AAD staining. PE, PE-Dazzle594, APC, and APC-Cy7 were detected through excitation using the 561 nm laser.

Conclusion

Using a NovoCyte flow cytometer equipped with a 561 nm laser allows better excitation efficiency of PE and PE-tandem fluorophores. It minimizes FITC spillover into the PE detection channel when PE is excited using the 561 nm laser, and provides additional flexibility by efficiently exciting various fluorescent proteins.

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