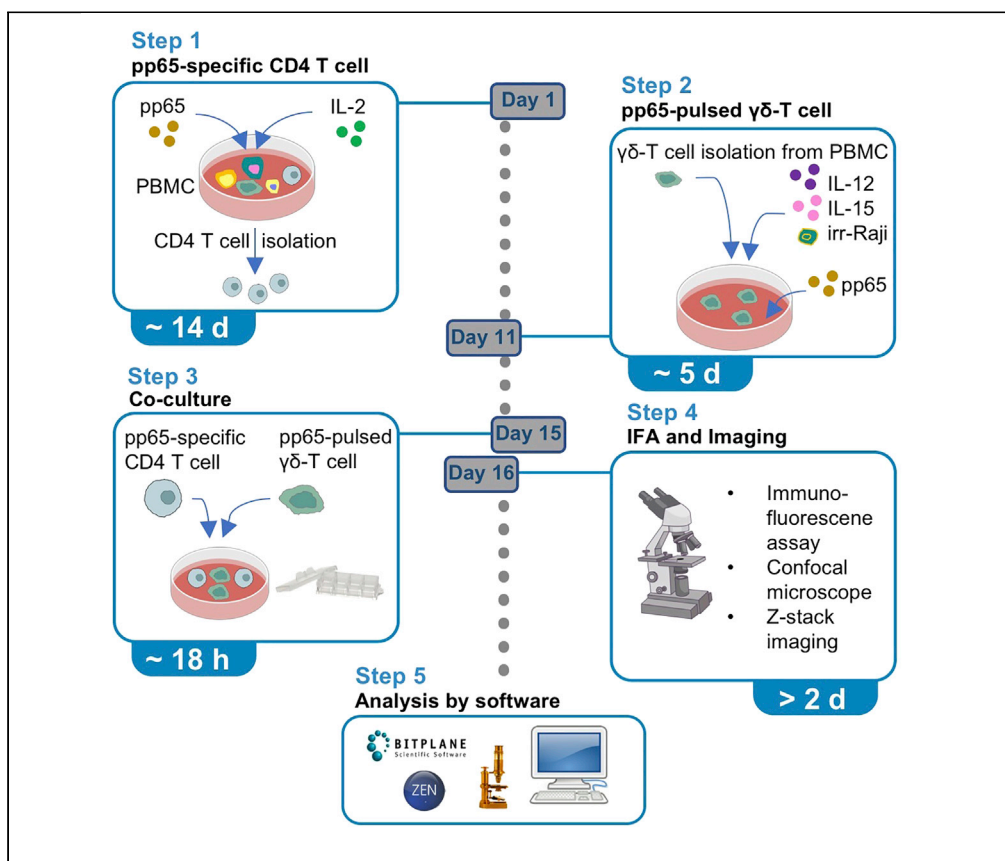


Protocol

Imaging and analysis on the interaction between human antigen-pulsed V δ 2 T cells and antigen-specific CD4 T cells



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Highlights
Protocol to co-culture antigen-pulsed V δ 2 T cell and antigen-specific CD4 T cell

Use of IFA for visualizing colocalization of proteins across cell-cell interface

Detailed procedures to perform Z-stack and 3D imaging analysis

This protocol describes how to visualize surface protein-protein co-localization across a cell-cell interface between antigen-presenting $\gamma\delta$ -T cells and CD4 T cells. By consolidating immunofluorescence assay, confocal microscopy, and 3D imaging analysis, it enables assessment of interaction between cell surface proteins such as Δ 42PD1 and TLR4 between co-cultured $\gamma\delta$ -T and CD4 T cells. This protocol can be applied to study a surface protein of interest and its potential interaction with a target cell/protein at the cell-cell interface.

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Protocol

Imaging and analysis on the interaction between human antigen-pulsed V δ 2 T cells and antigen-specific CD4 T cellsYufei Mo,^{1,3} Allen Ka Loon Cheung,^{2,3,4,*} Yue Liu,² Li Liu,¹ and Zhiwei Chen^{1,*}¹AIDS Institute and Department of Microbiology, State Key Laboratory of Emerging Infectious Diseases, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China²Department of Biology, Faculty of Science, Hong Kong Baptist University, Hong Kong SAR, China³Technical contact⁴Lead contact*Correspondence: akcheung@hkbu.edu.hk (A.K.L.C.), zchenai@hku.hk (Z.C.)
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SUMMARY

This protocol describes how to visualize surface protein-protein co-localization across a cell-cell interface between antigen-presenting $\gamma\delta$ -T cells and CD4 T cells. By consolidating immunofluorescence assay, confocal microscopy and 3D imaging analysis, it enables assessment of interaction between cell surface proteins such as Δ 42PD1 and TLR4 between co-cultured $\gamma\delta$ -T and CD4 T cells. This protocol can be applied to study a surface protein of interest and its potential interaction with a target cell/protein at the cell-cell interface. For complete details on the use and execution of this profile, please refer to Mo et al. (2020).

BEFORE YOU BEGIN

Generally, in this protocol, pp65-specific CD4 T cell and pp65-pulsed $\gamma\delta$ -T cell are co-cultured and visualized by confocal microscope. Figure 1 below displays the timeline for the whole protocol. All cells are to be incubated in 37°C/ 5% CO₂ cell culture incubator.

PBMC isolation

⌚ Timing: 2 h 30 min

1. Preparation before Gradient Centrifugation
 - a. Buffy coat blood from healthy donors (n=3) is collected under existing ethic approval protocols with local regulatory agencies.
 - b. Dilute buffy coat blood with sterile 1 × phosphate buffered saline (PBS) at a ratio of 1:3.
 - c. Load 30 mL diluted buffy coat gently in a 50 mL sterile conical centrifuge tube underlaid with 15 mL Ficoll-Paque.
 - i. Add 15 mL Ficoll-Paque to the bottom of a 50 mL centrifuge tube first.

Note: Temperature may affect Ficoll-Paque density, so it is important to keep Ficoll-Paque at room temperature (22°C~28°C) to ensure good performance on PBMC isolation.

- ii. When gently loading 30 mL diluted blood onto Ficoll-Paque, the pipetting speed should be as slow as possible at the beginning and increase gradually, in order to avoid heavily disturbing the Ficoll-Paque surface.



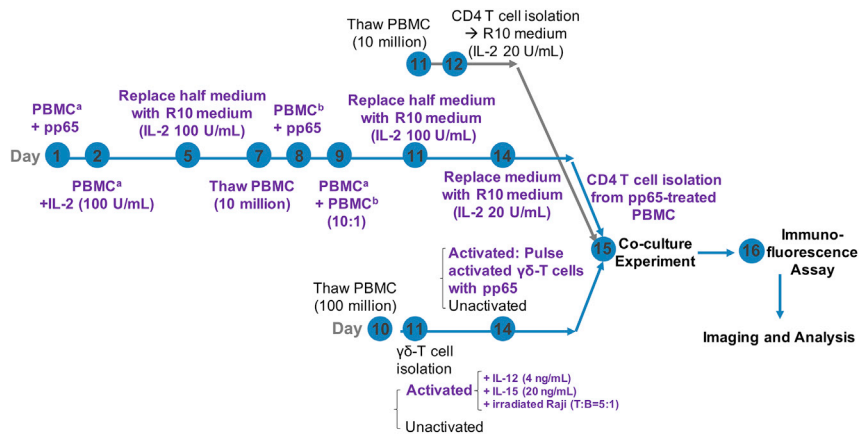


Figure 1. Timeline of the procedures described in this protocol

2. Gradient Centrifugation and Cell Collection

- a. Centrifuge at 652 g for 30 min.

△ CRITICAL: To ensure good performance, this procedure should be done at room temperature (22°C~28°C) due to the suitable Ficoll-Paque density. It is also important to keep acceleration at a slow rate (usually at 5) and brake at exactly 0.

- b. After centrifuging, the peripheral blood mononuclear cell (PBMC) layer would appear as a white yellow thin interface layer between the middle clear Ficoll-Paque layer and the upper yellow plasma layer.
- c. Use a Pasteur pipette to aspirate the PBMC layer and transfer into a new 50 mL sterile conical centrifuge tube.
- d. Makeup the volume to 45 mL with 1 × PBS and centrifuge the tube at 200 g for 10 min.
- e. The supernatant should be disposed by pouring into a waste container gently to avoid the loss of the cell pellet.
- f. Flick the bottom of the tubes to dislodge the pellet and resuspend the PBMC cell pellet with a total of 45 mL PBS and centrifuge again at 200 g for 10 min.
- g. Repeat e-f.
- h. Count the number of PBMC and check cell viability using 0.4% Trypan Blue and Hemacytometer under microscope.

Note: If the counting step takes less than 5 min, PBMC can be kept at room temperature (22°C~28°C). Otherwise, it would be better to keep cells at 4°C or on ice before further experiments. Normally, cell viability should be greater than 90% under good practice during PBMC isolation.

- i. Based on the cell count, remove 10 million PBMC for the immediate experiment (preparation of pp65-specific CD4 T cell) into a new tube.
- j. The remaining PBMCs can be centrifuged for a cell pellet and resuspended in freezing medium before use. Prepare the following tubes: one vial (100 million PBMC in 1 mL freezing medium) for $\gamma\delta$ -T cell isolation, one vial (10 million PBMC in 500 μ L freezing medium) for CD4 T cell isolation, while one vial (10 million PBMC in 500 μ L freezing medium) for PBMC supplementation in cell culture. The residual PBMC can also be stored (around 100 million PBMC in 1 mL freezing medium) as back-up when applicable.

Note: For short-term storage, cells can be stored at -80°C. For long-term storage, cells are suggested to be stored at -150°C.

Note: The protocol for isolating peripheral blood mononuclear cells is adapted from the recommended protocol from GE Healthcare, which is originally developed by Bøyum, A. (Bøyum, 1968).

Preparation of pp65-specific CD4 T cell

⌚ Timing: 14 days

3. pp65 Stimulation on Fresh PBMC

- On the day of PBMC isolation (Day 1), PBMC culture A (PBMC^a) is prepared. In order to stimulate pp65-specific memory CD4 T cells, resuspend 10 million PBMC in 2 mL R10 medium and supplement with 10 μ L PepTivator CMV pp65 (30 μ M). Distribute 1 mL of the cell suspension into each well of a 12-well plate.

Note: The expansion method of pp65-specific T cell is modified and adapted from the protocol published by other researchers (Loeth et al., 2012).

Note: This step is to obtain pp65-specific T cell for further co-culture experiment. Unstimulated CD4 T cell is isolated from frozen PBMC in step 4(a), serving as a negative control in the further experiment.

- On the next day (Day 2), add human IL-2 (working concentration at 100 U/mL) into the cell culture media.

Note: Two days after pp65 stimulation (on Day 3), cell clumps should be formed. The purpose of addition of IL-2 is to maintain the proliferation of activated pp65-specific CD4 T cell.

⚠ CRITICAL: If little or no clump is shown, it suggests that PBMCs from the donor might not be sensitive to pp65 or may not be CMV-seropositive, which should be excluded for the rest of the experiment.

- Replace half of cell culture media with fresh R10 medium supplemented with 100 U/mL IL-2 every 2–3 days until step 3(f). We recommend to do it on Day 5.
- On Day 7 (6 days after PBMC^a being prepared), recover one vial of frozen 10 million frozen PBMC (the same donor) in R10 medium pre-warmed to 37°C. PBMC culture B (PBMC^b) is prepared in this step.

Note: To ensure that the recovered PBMC will be healthy, it should be cultured in R10 medium overnight (18~24 h) before any next steps.

- On Day 8 [the next day after step 3(d)], resuspend 5 million PBMC^b with 1 mL R10 medium and supplement 5 μ L of PepTivator CMV pp65 in a 12-well plate.
- On Day 9 [the next day after step 3(e)], for the purpose of restimulation, add PBMC^b into PBMC^a culture at the cell number ratio of 1:10. Split each well of PBMC^a/PBMC^b mixture into two wells and add equivalent volume of R10 medium with 100 U/mL IL-2.
- Replace half of cell culture media with fresh R10 medium supplemented with 100 U/mL IL-2 every 2-3 days until step 3(h). We recommend to do it on Day 11 or Day 12.
- On the day before pp65-specific CD4 T cell isolation (Day 14), change PBMC culture media with fresh R10 medium supplemented with IL-2 at 20 U/mL to let cells rest.

4. Total CD4 T cell Isolation from Untreated PBMC and pp65-treated PBMC.

- On Day 11, recover frozen PBMC (the vial with 10 million PBMC for CD4 T cell isolation) in 5 mL R10 medium at the cell concentration at around 2 million cells per mL pre-warmed to 37°C. On the next day (Day 12), isolate CD4 T cell from recovered PBMCs using human total CD4 T cell isolation kit (negative selection), strictly according to manufacturer's instructions (<https://www.miltenyibiotec.com/DE-en/shop/comMiltenyiDatasheet/product?productid=130>).

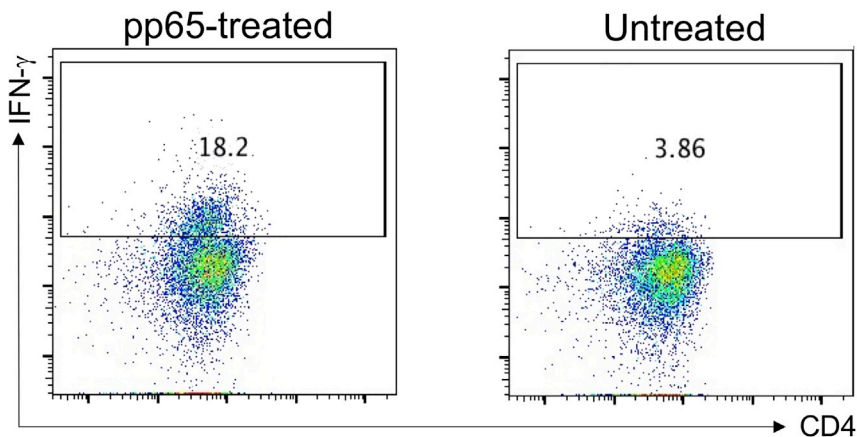


Figure 2. Examples for assessing IFN- γ -expression in CD4 T cell isolated from pp65-treated PBMC (left) and untreated PBMC (right) shown as dot plots by flow cytometry

Purified unstimulated CD4 T cells should be cultured in R10 medium supplemented with 20 U/mL IL-2 at cell density of 5 million per mL for around 3 days before co-culture.

△ CRITICAL: The purity of isolated CD4 T cell should be confirmed by FACS analysis using antibodies against CD3 and CD4.

Note: The duration of IL-2 treatment on total CD4 T cell isolated from untreated PBMC is optional from 2 to 4 days. The purpose of IL-2 treatment here is to maintain survival to avoid unpredicted effects of IL-2 on surface markers of CD4 T cell which may affect the interaction between $\gamma\delta$ -T cell and CD4 T cell. The cells here will serve as untreated control.

b. On the day of co-culture experiment (Day 15), pp65-treated PBMC can be harvested for total CD4 T cell isolation using the isolation kit mentioned in the above procedure (strictly according to manufacturer's instructions).

△ CRITICAL: The purity of isolated CD4 T cell should be confirmed by FACS analysis using antibodies against CD3 and CD4.

Note: According to the instruction of PepTivator CMV pp65, to purify IFN γ^+ CD4 $^+$ T cell by flow cytometer is recommended as a good way to purify pp65-specific CD4 T cell. However, the reason we could not do so is that we have to keep cells unlabeled from fluorescence-conjugated antibodies before confocal study. Technically, two-week culture with high dose of IL-2 may be good for activation and expansion of antigen-specific CD4 T cell. The percentage of IFN γ^+ in CD4 T cell isolated from pp65-treated PBMC can be monitored by FACS analysis using antibodies against CD3, CD4, and intracellular stained IFN- γ . An example of such FACS data is shown in [Figure 2](#).

Preparation of pp65-pulsed $\gamma\delta$ -T cell

⌚ Timing: 4 days

5. $\gamma\delta$ -T cell Isolation and Activation

- a. On Day 10, recover frozen PBMC (same donor as the above procedure, the vial containing 100 million PBMC) in 10 mL pre-warmed R10 medium and culture them at the concentration of 10 million cells per mL R10 medium overnight (for 18~24 h).
- b. On Day 11, isolate $\gamma\delta$ -T cell from recovered PBMCs using human total $\gamma\delta$ -T cell isolation kit (negative selection), strictly according to manufacturer's instructions

(<https://www.miltenyibiotec.com/DE-en/shop/comMiltenyiDdatasheet/product?productId=200>). Purified $\gamma\delta$ -T cell is left untreated or treated with IL-12 (4 ng/mL) and IL-15 (20 ng/mL) in the presence of 70 Gy γ -irradiated Raji B cell at the ratio of (T:B = 5:1) at a total cell concentration at 1~2 million cells per mL for 18–72 h (at least 18 h for activation) to express the surface proteins such as $\Delta 42PD1$ and HLA-DR.

△ CRITICAL: The purity of isolated $\gamma\delta$ -T cell ($CD3^+V\delta 2^+$) and activation phenotype (expression of $\Delta 42PD1$ and HLA-DR) should be confirmed by FACS analysis.

Note: Similar to other studies that used irradiated Epstein-Barr virus (EBV)-transformed B cells or primary B cells (Brandes et al., 2005, Brandes et al., 2003) as feeder cells for CD40/CD40L interaction, these cells can induce an increased upregulation of co-stimulatory molecules such as HLA-DR, CD40, CD80 and CD86 on $V\delta 2^+$ cells during soluble antigen presentation process (Brandes et al., 2005, Himoudi et al., 2012). One type of EBV-transformed B cells, Daudi cells (Himoudi et al., 2012), could directly stimulate the $V\delta 2^+$ cells for additional functions. In contrast, Raji B cell line is not directly stimulatory to $V\delta 2^+$ cells (L'Faqihi et al., 1999, Fisch et al., 1990). In our experiment, we aim at investigating the antigen presenting role of $\Delta 42PD1^+V\delta 2^+$ cells induced by IL-12 and IL-15, without additional stimulation by feeder cells (such as Daudi cells), so Raji B cells are the suitable EBV-transformed B cell line here. Importantly, in our experiments Raji cells were γ -irradiated prior to co-culture with antigen-pulsed $\gamma\delta$ -T cells and therefore would not respond to stimulation or produce new cytokines to affect CD4 T cells in the co-culture.

6. pp65-pulsed antigen presenting $\gamma\delta$ -T cells
 - a. Three days after IL-12/IL-15 treatment (on Day 14), pulse 5 million IL-12/IL-15 activated $\gamma\delta$ -T cells with 5 μ L of PepTivator CMV pp65 (30 μ M) in 1 mL culture for 24–36 h before co-culture experiment (details of co-culture experiment are displayed in [step-by-step method details](#)).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti- $\Delta 42PD1$ antibody (clone: CD34), mouse IgG2b (See note below)	In-house	N/A
TCR V delta 2 Monoclonal Antibody (clone: 15D), mouse IgG1	Thermo Fisher	Cat# TCR1732 RRID:AB_417090
TLR4 Antibody (clone: HTA125), mouse IgG2a	Santa Cruz	Cat# sc-13593 RRID:AB_628366
TLR4 Antibody (clone: 25), mouse IgG1	Santa Cruz	Cat# sc-293072 RRID:AB_10611320
Purified anti-human CD4 Antibody (clone: RPA-T4), mouse IgG1	BioLegend	Cat# 300502 RRID:AB_314070
HLA-DR Antibody (clone: L243), mouse IgG2a	Novus Biologicals	Cat# NB100-77855 RRID:AB_1085035
Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	Cat# A-21121 RRID:AB_2535764
Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Invitrogen	Cat# A-21134 RRID:AB_2535773
Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	Cat# A-21242 RRID:AB_2535811
Biological samples		
Buffy coat from healthy donors	Hong Kong Red Cross (See note below)	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Human IL-12	Miltenyi Biotec	Cat# 130-096-705
Human IL-15	Miltenyi Biotec	Cat# 130-095-765
Recombinant Human IL-2 Protein	R&D Systems	Cat# 202-IL-050
PepTivator CMV pp65, human	Miltenyi Biotec	Cat# 130-093-438
Ficoll-Paque	GE Healthcare	Cat# 17-5442-03
Fetal bovine serum	Gibco	Cat# 10270106
PBS (10x), pH 7.4	Gibco	Cat# 70011069
Bovine serum albumin [Fraction V Bovine Albumin, pH 7]	Thermo Scientific	Cat# AAJ1085722 CAS: 9048-46-8
DMSO	Sigma	Cat# D2650-100ML CAS: 67-68-5
L-Glutamine (200 mM)	Gibco	Cat# 25030081
Penicillin-streptomycin (10,000 U/mL)	Gibco	Cat# 15140122 CAS: 3810-74-0 (Streptomycin)
Paraformaldehyde	Sigma	Cat# 158127-100G CAS: 30525-89-4
Triton™ X 100	Sigma	Cat# X100-500ML CAS: 9002-03-1
Trypan blue solution, 0.4%	Life Technologies	Cat# 15250061 CAS: 72-57-1
UltraPure™ DNase/RNase-free Distilled Water	Invitrogen	Cat# 10977015
RPMI 1640 Medium, no glutamine	Gibco	Cat# 21870092
Critical commercial assays		
CD4+ T Cell Isolation Kit, human	Miltenyi Biotec	Cat# 130-096-533
TCRγ/δ+ T Cell Isolation Kit, human	Miltenyi Biotec	Cat# 130-092-892
Experimental models: cell lines		
Raji B cell	Kindly provided by Prof Honglin Chen (HKU).	N/A
Software and algorithms		
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
JACoP plugin for ImageJ	(Bolte and Cordelières, 2006)	http://www.blackwell-synergy.com/doi/pdf/10.1111/j.1365-2818.2006.01706.x
ZEN Blue	Zeiss	v2.3
Imaris	Oxford Instruments	v7.5.2
Other		
Nunc™ Lab-Tek™ II 8-well chambered coverglass	Thermo Fischer	Cat# 155409
SlowFade™ Gold antifade mounting medium with DAPI	Invitrogen	Cat# S36938
Carl Zeiss LSM 700	Zeiss	N/A
Carl Zeiss LSM 800	Zeiss	N/A
Leica TCS SP5 II	Leica	N/A
Centrifuge 5810R	Eppendorf	N/A
BD FACSAria™ III Cell Sorter	BD Biosciences	N/A
MDS Nordion Gammacell 3000 Elan	Best® Theratronics	N/A

Note: Anti-Δ42PD1 antibody (clone: CD34, Mouse IgG2b) is available upon request.

Note: Buffy coat from healthy donors can also be obtained by other sources appropriate for the researchers' country, districts or areas with ethics approval.

Note: The antibodies for FACS analysis in those critical points (assessing purity or activation) are optional and depend on the user, so they are not included here.

Optional: Other imaging processing software may be used for data analysis.

MATERIALS AND EQUIPMENT

R10 medium	Final concentration	Volume (mL)
RPMI 1640 medium	N/A	440
FBS	10%	50
L-glutamine (200 mM)	2 mM	5
Penicillin and streptomycin (10000 U/mL and 10 mg/mL, respectively)	100 U/mL and 100 µg/mL, respectively	5
Total		500

Once prepared, it should be stored at 4°C and used within 4 weeks.

Freezing medium	Final concentration	Volume (mL)
FBS	90%	9
DMSO	10%	1
Total		10

It should be freshly prepared before use.

Stock of PepTivator CMV pp65	Final concentration	Amount
PepTivator CMV pp65	N/A	6 nmol
Sterile UltraPure™ DNase/RNase-free Distilled Water	N/A	0.2 mL
Total		0.2 mL

Once prepared, it should be aliquoted into 10 µL per tube and frozen at –80°C for one year. Repeated freeze-thaw cycles should be avoided.

Perm/Block buffer	Final concentration	Amount
PBS	N/A	9.45 mL
FBS	5%	0.5 mL
Bovine Serum Albumin (BSA)	3%	0.3 gram
Triton-X 100	0.5%	0.05 mL
Total		10 mL

Once prepared, it should be stored at 4°C and used within 4 weeks.

Staining Buffer	Final concentration	Amount
PBS	N/A	49.95 mL
Bovine Serum Albumin (BSA)	3%	1.5 gram
Triton-X 100	0.1%	0.05 mL
Total		50 mL

Once prepared, it should be stored at 4°C and used within 4 weeks.

STEP-BY-STEP METHOD DETAILS

Co-culture of pp65-specific CD4 T cell and pp65-pulsed $\gamma\delta$ T cell

⌚ Timing: 2 h

In this step, CD4 T cell isolated from pp65-stimulated PBMCs is co-cultured with pp65-pulsed $\gamma\delta$ -T cell before proceeding to immunofluorescent assay. It is Day 1 for co-culture experiment, as well as Day 15 for the whole experimental procedure.

1. Co-culture

- a. Nunc™ Lab-Tek™ II 8-well chambered coverglass is used for the co-culture experiment.

Note: The Nunc™ Lab-Tek™ II 8-well chambered coverglass is made up of cell-culture treated glass bottom chambers, which allowed our cells to adhere to the bottom of chamber after overnight culture. Since we sought to analyze the cell-cell interaction, the situation that cells tightly adhere to the chamber bottom before cell-cell interaction should be avoided. Therefore, we do not recommend using coating reagents facilitating cell adherence, for instance, fibronectin and poly-L-lysine.

- b. Co-culture CD4 T cell isolated from pp65-stimulated PBMCs or unstimulated PBMCs with pp65-pulsed activated or unactivated $\gamma\delta$ -T cell, at a ratio of $\gamma\delta$ -T:CD4 T = 1:1 (a total of 0.2 million cells in 200 μ L R10 medium per well) overnight (around 18 h). Thus, eight groups are involved:

Group	Cell culture	Aim
1	Unactivated $\gamma\delta$ -T cell	Unactivated cell only control
2	pp65-pulsed activated $\gamma\delta$ -T cell	Antigen presenting cell only control
3	Unstimulated CD4 T cell	Effector cell negative control
4	pp65-stimulated CD4 T cell	Effector cell only control
5	Unactivated $\gamma\delta$ -T cell plus unstimulated CD4 T cell	Negative control
6	Unactivated $\gamma\delta$ -T cell plus pp65-stimulated CD4 T cell	Internal control
7	pp65-pulsed activated $\gamma\delta$ -T cell plus unstimulated CD4 T cell	Internal control
8	pp65-pulsed activated $\gamma\delta$ -T cell plus pp65-stimulated CD4 T cell	Test

Note: The recommended culture volume in the Nunc™ Lab-Tek™ II 8-well chambered coverglass is 0.2–0.5 mL, which is capable of culturing 0.2 million to 0.5 million cells at the density of ~1 million cells per mL. To assist the cells to settle at the bottom of the well, culture volume lower than 0.4 mL would be better. Thus, the maximal cell number per well should be 0.4 million in 0.4 mL culture.

Note: In total, 24 wells (8 groups \times 3 donors) are needed. Cells are cultured in a 37°C/5% CO₂ incubator overnight (around 18 h).

△ **CRITICAL:** For the sake of seeing specific conjugates, co-culture of “unstimulated CD4 T cell and pp65-pulsed $\gamma\delta$ -T cell” and co-culture of “pp65-specific CD4 T cell and unactivated $\gamma\delta$ -T cell” should be included as control.

△ **CRITICAL:** Spare one or two more wells serving for the secondary antibody control in the immunofluorescence assay.

Immunofluorescence assay

⌚ **Timing:** 2 days

$\gamma\delta$ -T cells and CD4 T cells are allowed to interact after 18-h co-culture, cells are then subjected to immunofluorescence assay (IFA) and evaluated with confocal microscopy (Day 15 overnight to Day 16, for the whole experimental procedure).

- Following overnight (~18 h) co-culture, cells are now ready for antibody staining.
- On Day 1 for immunofluorescence assay, using a 200 μ L pipette, aspirate the media slowly and carefully from top-to-bottom at the bottom-right corner of each well.

△ **CRITICAL:** After overnight culture, these suspension cells should be settled down on the bottom of each well though they are not attached tightly, as mentioned above. In order to avoid lifting of the cells and to minimize cell loss, the procedure of moving the chamber, aspirating or adding any buffer should be done slowly and carefully along the sides of the wells. For consistency, the down-right corner of each well should be fixed for removing the media or PBS, while the upper-left corner of each well is used to add buffer, in a slow and gentle manner. This applies for the rest of the following steps.

4. Add 200 μ L PBS slowly and gently to each well at the upper-left corner.
5. Aspirate the PBS slowly and carefully from top-to-bottom at the bottom-right corner of each well.
6. Add 200 μ L of 4% paraformaldehyde (PFA, dissolved in PBS, pH 7.4) to each well and keep it at room temperature (22°C~28°C) in the dark for 20 min to fix the cells.
7. Aspirate the 4% PFA and wash with 200 μ L PBS twice.
8. Add 200 μ L of Perm/Block buffer and keep the chamber at 4°C for 20 min.
9. Wash with 200 μ L Staining Buffer twice.
10. Incubate with primary antibodies in 200 μ L Staining Buffer at 4°C overnight (~18 h). For example, in our experiment, we used the following primary antibodies:
 - a. Panel 1: anti- Δ 42PD1 (CH34, Mouse IgG2b, 1:100), anti-V δ 2 (15D, Mouse IgG1, 1:20), and anti-TLR4 (HTA125, Mouse IgG2a, 1:20).
 - b. Panel 2: anti- Δ 42PD1 (CH34, Mouse IgG2b, 1:100), anti-HLA-DR (L243, Mouse IgG2a, 1:250), anti-TLR4 (25, Mouse IgG1, 1:50)

△ **CRITICAL:** For non-specific signal control, do not use primary antibodies and only add appropriate secondary antibodies.

11. On the next day (Day 2 for immunofluorescence assay), wash with 200 μ L Staining Buffer twice.
12. Incubate with secondary antibodies in 200 μ L Staining Buffer at 4°C for 30 min. In our experiment, we used the following secondary antibodies for the above both panels:
 - a. Goat anti-mouse IgG1 AF488 (1:1000), goat anti-mouse IgG2a AF568 (1:1000) and goat anti-mouse IgG2b 647 (1:1000).
13. Wash with 200 μ L Staining Buffer twice.
14. Mount the cells by adding 200 μ L SlowFade™ Gold antifade reagent with DAPI, and incubate at room temperature (22°C~28°C) in the dark for 30 min.

△ **CRITICAL:** Unlike coverslip/microscope slides, the chamber is not needed to be turned upside-down for imaging, as it is imaged from the bottom of the chamber. No coverslip is needed here as well. However, sufficient volume of the mounting medium (200 μ L) is important to fully cover the cells and to prevent evaporation.

Optional: If mounting with SlowFade™ reagent without DAPI, cells can be stained with DAPI in 200 μ L PBS at room temperature (22°C~28°C in the dark; incubation time depends on the instruction of different commercial products) and then washed with 200 μ L PBS before applying mounting medium.

15. Keep the chamber with the lid at 4°C before imaging under confocal microscope.

Note: It is better to perform imaging as soon as possible to get clearer signals without bleaching, within one month after IFA is done.

▣ **Pause point:** The chamber can be kept in dark place at 4°C, placed in a sealed container or glad wrapped, for the purpose of protecting from light and from drying, if performing confocal microscopy at a later time.

3D imaging using confocal microscope

Ⓞ **Timing:** Depends on availability of the confocal microscope at your institution and familiarity of operation, it may take multiple sessions of several hours to obtain sufficient data.

In this step, IFA samples can be imaged using confocal microscopes such as LSM700, LSM800 and Leica SP 5 II. We mainly focused on the colocalization of the signal of $\Delta 42PD1$ on $V\delta 2^+$ cell and the signal of TLR4 on CD4 T cell, as well as the presence of HLA-DR on $V\delta 2^+$ cell. The protein expression on the cell only groups (Group 1, 2, 3 and 4) serve as controls.

16. The pixel resolution for the different objective lens used will depend on the specific parameters and the confocal microscope being used.

Note: The details about the objectives we used are shown below:

Magnification	Numerical aperture	Free working distance	Coverglass thickness	Immersion
10×	0.3	2 mm	0.17 mm	Air
40×	1.4	0.13 mm	0.17 mm	Oil
63×	1.4	0.19 mm	0.17 mm	Oil

To acquire images with high quality, we recommend using the optimal setting of ZEN or relevant acquisition software during image capturing.

17. Scan at least 3 images for each group using 10× lens first.

Note: Scanning images using 10× lens first is helpful to have a general view of the distribution of signals and to identify the cells of interest, which is a preliminary step before locating the scanning position using 40× or 63× lens.

18. For experimental group 5 (unactivated $\gamma\delta$ -T cell plus unstimulated CD4 T cell), group 6 (unactivated $\gamma\delta$ -T cell plus pp65-stimulated CD4 T cell), group 7 (pp65-pulsed activated $\gamma\delta$ -T cell plus unstimulated CD4 T cell) and group 8 (pp65-pulsed activated $\gamma\delta$ -T cell plus pp65-stimulated CD4 T cell), scan at least five images per chamber using 40× or 63× oil lens. Scan at least five Z-stack images for group 8.

Note: A resolution of at least 1024*1024 is recommended but depends on the objective lens used. User should check the hardware parameters.

Note: Sufficient number of images is helpful in a robust data quantification and analysis afterwards.

Note: T-PMT should be turned off and the bright field should be excluded during Z-stack imaging. Setting the Z-position: decrease focus height until you cannot see the cell structure and set this as the first scanning position of the Z-position and increase focus height until you cannot see the cell structure and set as its last scanning position. Click "1 AU" hotkey to adjust pinhole size. Use the recommended parameters on frame size and resolution (click "Optimal" option) by the software. Use the scan speed recommended by the software.

Optional: The condition we set is that Z-stack interval is 0.506 μm with 25 images acquired. The interval should be smaller than one airy unit. Smaller interval leads to slower scanning but higher resolution along the Z-axis. It should be adjusted to the resolution limit of the

objective lens used. The number of acquired images depends on both the length of Z-position and the interval.

EXPECTED OUTCOMES

At the end of this protocol the protein-protein co-localization on the interface between antigen-presenting $\gamma\delta$ -T and CD4 T cells can be visualized by 3D imaging. Data generated using this protocol has recently been published (Mo et al., 2020), as shown in [Figure 3](#).

Generally, the technique of image acquisition through Z-stack provides us important data of the cell-cell interface. Further data analysis through ImageJ provides valuable quantitative results on the co-localization coefficients values and signal profile across interface. Imaris analysis also constructs a 3D model for the interface of interest.

QUANTIFICATION AND STATISTICAL ANALYSIS

Determining co-localization coefficients

To determine whether two proteins are expressed at the same location, co-localization coefficients are calculated using the JACoP plugin in ImageJ.

1. Choose an image taken using high magnification such as 630 \times and open using ImageJ.
2. Identify the cells of that appears to be interacting and crop the image to the particular area. Save this image.
3. Split the image into individual channels, such that one image only displays one parameter. Save the individual images.

Note: In our study, we aimed to determine the co-expression of $\Delta 42$ PD1 and HLA-DR. Thus, the confocal image was split into individual images displaying $\Delta 42$ PD1, HLA-DR, DAPI, and brightfield channels.

4. Choose the two channels for analysis in JACoP, e.g., $\Delta 42$ PD1 for Image A and HLA-DR for Image B. Save these images.
5. In the analysis to perform, choose the ones appropriate to you. Our analysis used Overlap and Manders' coefficients.

Note: Overlap coefficient is based on Pearson's coefficient that is dependent on linear signals while Manders' coefficient take into account of ratios of two signals on top of the compartment and vice versa. Having both coefficients will provide a more comprehensive co-localization analysis.

6. In the Threshold tab, adjust the slider for channel A and B to the level of signals that can clearly differentiate between background without signal saturation. Press Analyze.
7. In the data log window in ImageJ, record the Overlap coefficient and Manders' coefficients (using threshold value, for both A overlapping B and B overlapping A).
8. Using steps 3–7, analyze at least 50 cells per experimental group across multiple images.
9. Comparisons can be made in a table or as a graph where statistics can be calculated.

Determining the co-localization between two surface proteins across a cell-cell interface

10. Choose an image taken using high magnification such as 630 \times and open using ImageJ ([Figure 4A](#)).
11. Identify the cells that are in close proximity and expressing the protein interaction of interest. Crop the image to the particular cell. Save this image ([Figure 4A](#)).

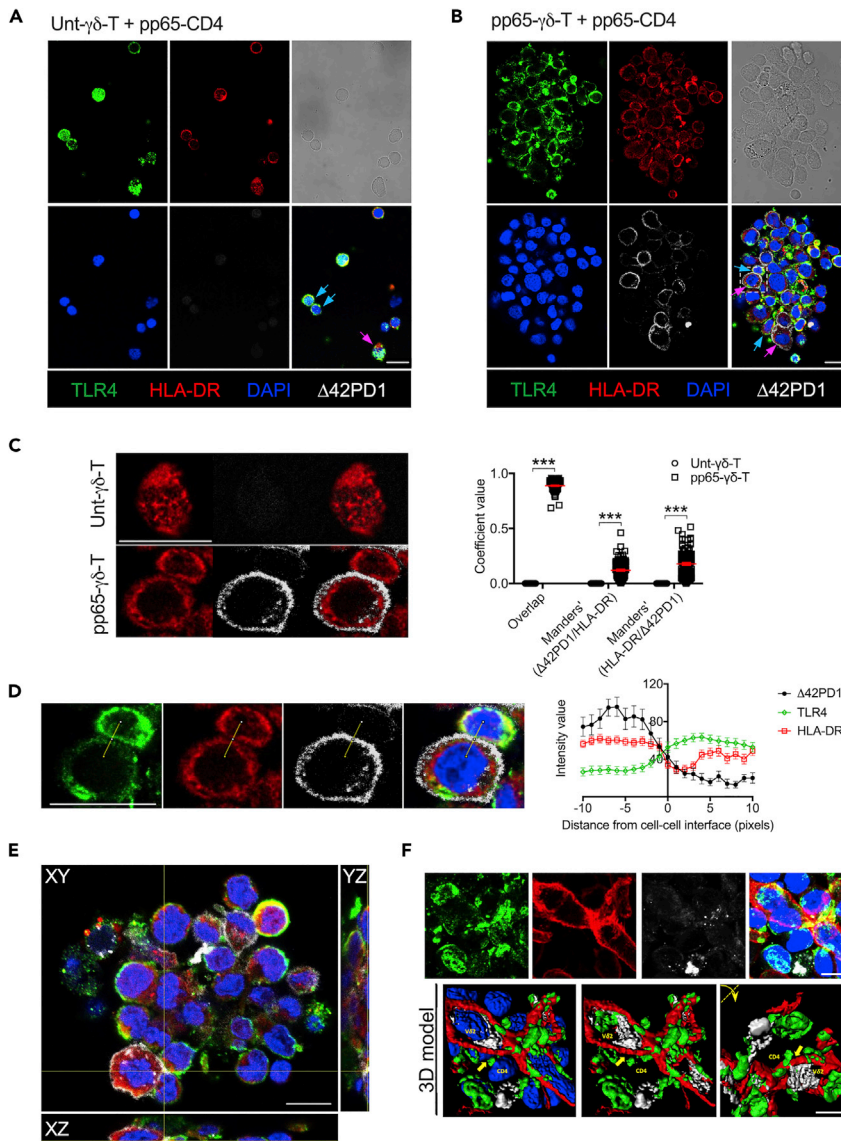


Figure 3. Example of data from using this protocol describing $\Delta 42PD1$ -TLR4 interaction between $V\delta 2$ and $CD4^+$ T cells

(Figure reprinted with permission from Mo et al., 2020). pp65-specific $CD4^+$ T cells were used to co-culture with autologous IL-12/IL-15 stimulated and pp65-pulsed $\gamma\delta$ -T cells or untreated (Unt) $\gamma\delta$ -T cells for around 18 h, before fixed and immunostained for $\Delta 42PD1$ (white), TLR4 (green), HLA-DR (red) and DAPI (blue). Co-culture of pp65-specific $CD4^+$ T cells with (A) unstimulated $\gamma\delta$ -T cells or (B) with IL-12/IL-15 stimulated and pp65-pulsed $\gamma\delta$ -T cells. Pink and blue arrows point to $\Delta 42PD1^+$ HLA-DR $^+$ and TLR4 $^+$ cells, respectively. Pixel size = 120.2 nm.

(C) Representative images showing colocalization of $\Delta 42PD1$ and HLA-DR expression signals on Unt ($n = 65$) and pp65-pulsed $\gamma\delta$ -T cells ($n=96$) were analyzed, for the overlap and Manders' coefficient values shown in the graph.

(D) Representative images showing the interaction between pp65-specific $CD4^+$ T cell and pp65-pulsed $\gamma\delta$ -T cells analyzed by the signal values for $\Delta 42PD1$, MHC-II and TLR4 along the yellow line transecting from bottom left to top right equivalent to left to right in the histogram. Values are indicated by the histogram of $n=32$ cell-cell pairs at ten pixels before and after interface between the cells set at $x = 0$.

(E) Orthogonal views over XY, XZ and YZ axes of a Z-stack of 78 images at 0.29 μm thickness each. Image at stack 72 is shown for the interaction between pp65-specific $CD4^+$ T cell and pp65-pulsed $\gamma\delta$ -T cells. Pixel size = 80.2 nm.

(F) Imaris analysis on a maximum projection of a Z-stack of 25 images each with a thickness of 0.506 μm (upper) to generate the 3D model (bottom). Pixel size = 112 nm. The rightmost image is a flipped version of the middle image to indicate interactions at two different angles. White bar represents a scale of 10 μm . Representative images from three independent experiments are shown. Data in the graphs represent mean \pm SEM.

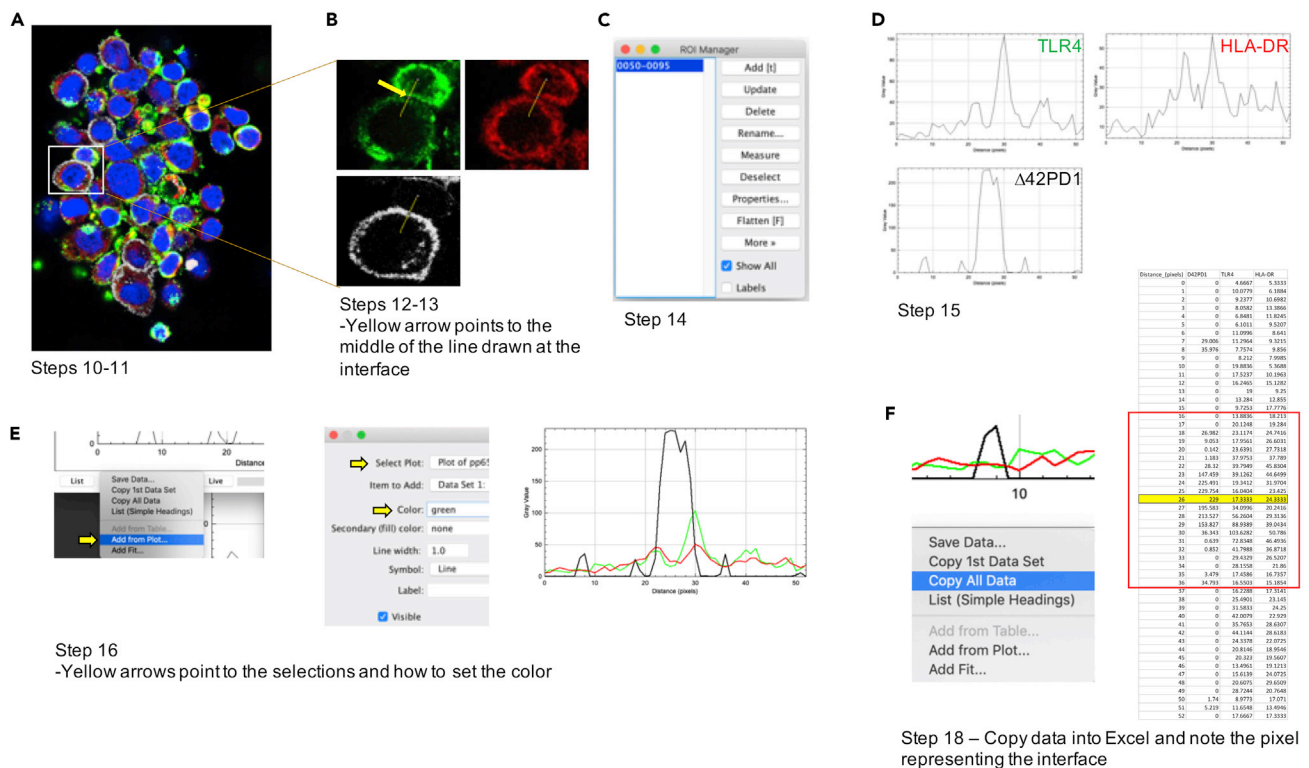


Figure 4. Procedures to analyze pixel profiles across a cell-cell interface

(A) Example of choosing a cell-cell interface area of interest. (B) Drawing a line of interest across the interface where yellow arrow points to the middle of the line. (C) Adding the line of interest in the ROI Manager. (D) Profile plots of the different protein expression across the line. (E) Overlaying the profile plots. (F) Extracting the pixel data from the profile plots.

- Split the image into individual channels, such that one image only displays one parameter. Save the individual images (Figure 4B).
- Using the drawing tool, draw a line across the membrane of two cells beginning and ending in the respective cytoplasmic regions. Make sure the middle of the line (usually displayed as a square dot) is at the middle of cell-cell interface. Draw for one image first (Figure 4B).
- Go to Analyze\Tools\ROI Manager then click on "Add", this will designate a number for the line. Click on the images showing the other individual channels and click the newly added line number in ROI Manager to apply the same line to the other images (Figure 4C).
- Click on the channels of interest with line drawn, then go to Analyze\Plot Profile to show the signals for the individual channels (Figure 4D).
- In one of the plots, click the "Data" button and choose "Add from Plot" for the other channels with appropriate colors (typed the color you desire, e.g., "green", "red") (Figure 4E).

Note: In our study, we wanted to examine the interaction between $\Delta 42PD1$ /HLA-DR on pp65-pulsed activated $\gamma\delta$ -T cell and TLR4 on pp65-stimulated CD4 T cell. Thus, the plot included signals for $\Delta 42PD1$, HLA-DR and TLR4 across the line drawn between the $\gamma\delta$ -T cell and CD4 T cell.

- Repeat these steps to analyze additional cell-cell pairs.
- Data from the plots can be exported for the different signals from one cell-cell pair by clicking "Data" then "Copy all data" and paste into Excel worksheet or other softwares (Figure 4F).

Note: The data will depict the signal value of each pixel across the line that is drawn across the cell-cell interface. However, the length of the line can vary, and it is why in step 13, the middle

square dot of the line should be placed in the middle of the interface. Then only the signal values of 10 pixels forward and backward from the middle square dot should be used across the different cell-cell pairs and different line lengths. Taking the example in [Figure 1](#), the line is 0 to 52 pixels in length, so the middle square dot sits at pixel 26, and the data from 16 to 36 should be included for analysis.

Note: At least 30 cell-cell pairs are recommended for drawing the line, generating plot profiles, and for data analysis.

Examining the cell-cell interface in Z stack and orthogonal views

The purpose of viewing a Z-stack in an orthogonal view is to identify cell-cell and protein-protein colocalization at different angles. There could be interactions that occur in an up-down orientation that would be missed if only the XY-plane is considered. Further, any interactions found on one plane should be confirmed in another plane and the orthogonal views will enable this.

19. Choose a Z-stack taken using high magnification such as 630× and open using ImageJ.
20. Identify the cells that are in close proximity and expressing the protein interaction of interest. Crop the Z-stack to display the desired cell-cell interaction. Save this Z-stack.
21. Using ImageJ, click on Image\Stacks\Orthogonal views. The YZ and XZ images will be displayed and can see to the cell-cell and protein-protein interaction at different angles. Save the displayed images.
22. Move the crosshair to highlight the interaction at the different stack, which could be used for analysis as above.

Generating a 3D model for cell-cell and protein-protein interaction using Imaris

Imaris is a useful image analysis software on generating a visual 3D model for protein-protein interaction among cells.

23. Import Z-stack files generated from LSM700 into Imaris software, as shown in [Figure 5A](#).
24. Select "Surpass" mode → check the channel that needs to be processed and uncheck others ([Figure 5B](#)) → tick "Light Source 1" and "Volume" → click "Add new surface" ([Figure 5C](#)) and define color for each channel [color can be set by experimenter in the "color" column in [Figure 5D](#), and it is not limited to the default one.] → set the optimal algorithm (using default parameters), source channel ([Figure 5E](#)), threshold ([Figure 5F](#)) and number of voxels ([Figure 5G](#)) → process and obtain the virtual image of one channel ([Figure 5H](#)). → Process other channels using similar procedures.

Note: Although DAPI represents the cell nuclear, we still choose the "surface" mode for it, which may show the surface of nuclear.

Note: To make those surfaces better visualize, the optimal threshold and number of voxels should be adjusted. The adjustment is supposed to exclude noise signals but cover all the clear signals. Too high threshold leads to large noise ([Figure 6A](#)), while too low one leads to failure of covering signals ([Figure 6B](#)). [Figure 6C](#) shows the suitable threshold which covers most of bright signals. As for number of voxels, too high number of voxels ([Figure 6D](#)) fails in exclude noise signals, but too low number of voxels ([Figure 6E](#)) is not able to fully cover those clear signals. [Figure 6F](#) shows the optimal number of voxels that excludes noise and that makes a suitable coverage of bright signal.

25. After virtual images of all channels are processed, check the processed surface programs and uncheck the raw signals, obtain one 3D model for the interface, as shown in [Figure 7](#).

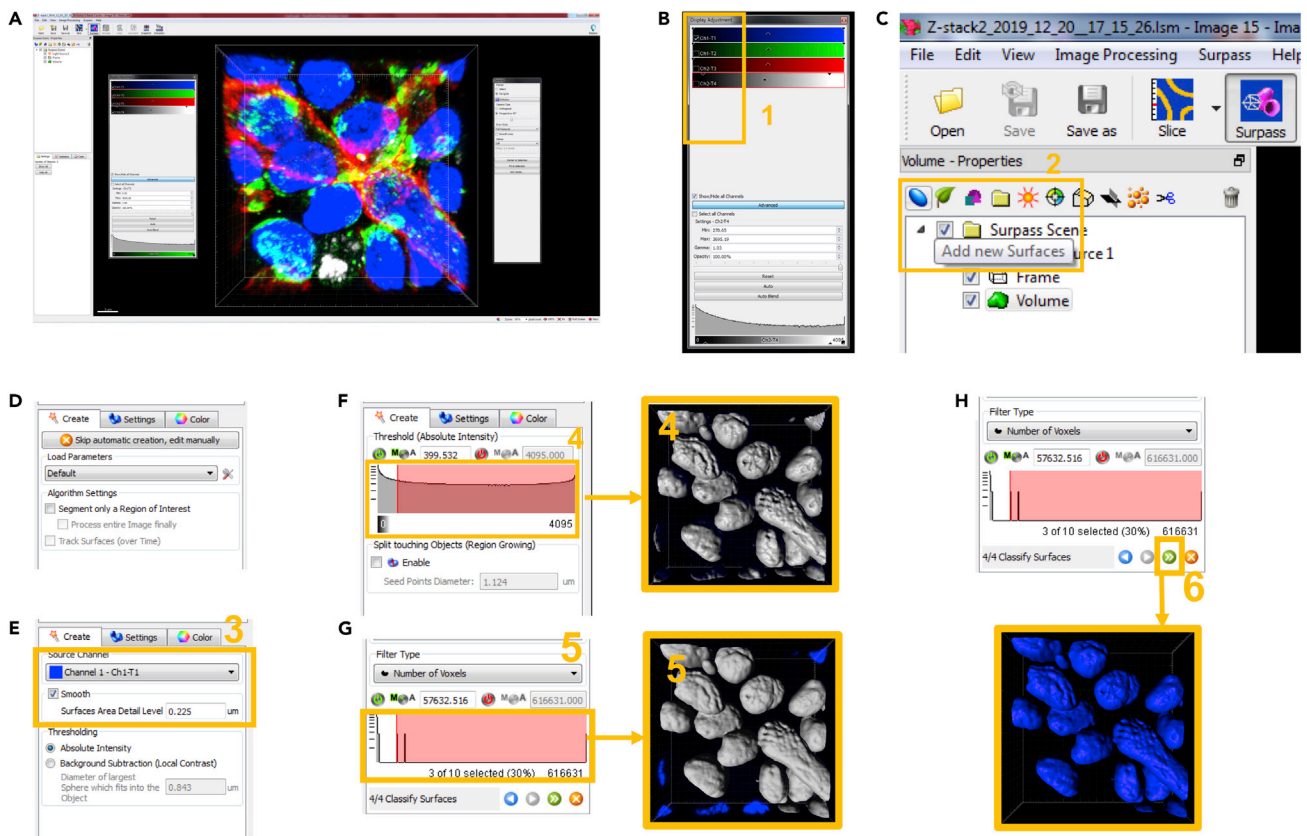


Figure 5. Procedures for how to process virtual image from one channel

(A) Opening the Z-stack file of interest in the working space of Imaris software. Different channels (B) and functional options (C) in the working space are shown in enlarged display. (D) General settings on parameters and color before proceeding to algorithm creation. Channel (E), threshold (F) and number of voxels (G) can be selected during algorithm creation. (H) Algorithm can be processed in order to obtain the virtual image of one specific channel.

LIMITATIONS

Although we showed an applicable protocol on displaying the protein co-localization on the cell-cell interface, an ideal model for cell-cell interaction is to do live cell imaging on the cell-cell co-culture system. However, since the biomarker antibody also serves as blocking antibody and the two clones of in-house antibodies have the same problem, it is difficult to image cell-cell interface. To counteract with this problem, more clones of antibody for the specific biomarker, not limited to the antibodies we used for our research, can be considered to develop for different experimental purpose separately. It should be addressed that the antibodies under investigation would not block the interaction between the proteins of interest and would be able to access between cells of interest, which is a long and difficult way to develop.

In terms of problems involving signal strength in the confocal study, conjugated primary antibodies may provide relatively weak signal. Labeling of the proteins using unconjugated primary antibody followed by conjugated secondary antibody allows amplification of signals. It will increase signal-to-noise ratio, while using conjugated primary antibodies may have reduced signal-to-noise ratio. Under this limitation, we chose the primary/secondary antibodies approach to ensure signal strength. We should also be careful about determining the optimal concentration of primary and secondary antibodies in the immunofluorescence assay.

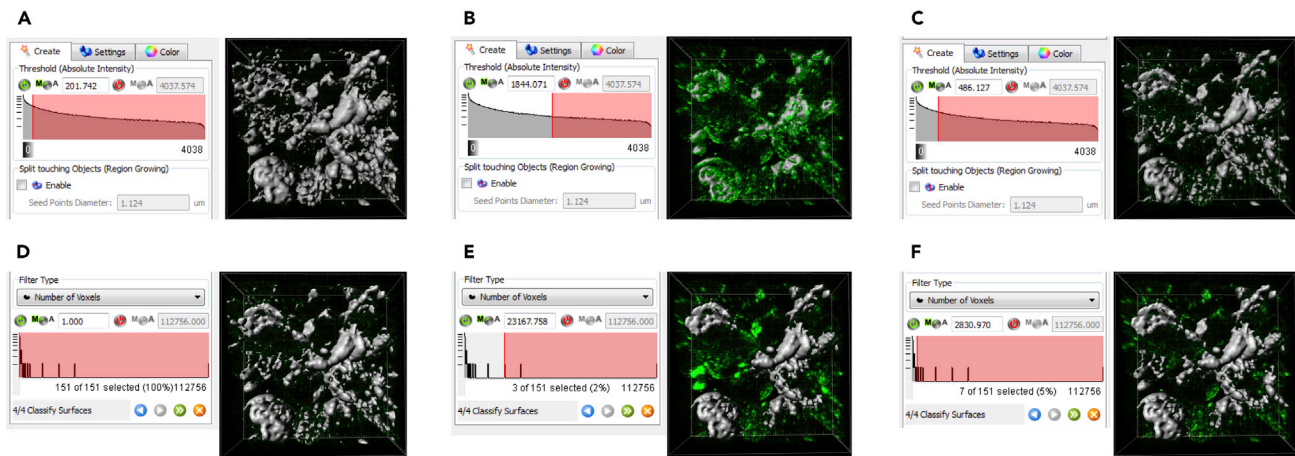


Figure 6. Comparison on results of high, low or optimal level of threshold and number of voxels

The results of high threshold (A), low threshold (B) and optimal threshold (C) are displayed. The results of high number of voxels (D), low number of voxels (E) and optimal number of voxels (F) are displayed.

TROUBLESHOOTING

Problem 1

Cell loss during IFA procedure (steps 1, 3, 4, and 17 in [step-by-step method details](#)).

Potential solutions

Solution 1. Total cell number seeded in each assay well should not be less than 0.2 million cells or larger than 0.4 million cells per well, given the appropriate culture volume of 0.2–0.4 mL. If experimenter can not find enough cells for imaging or experimenter suffer from cell loss during IFA procedure, total cell number can reach around 0.4 million. However, too high density of cell number may not be good for regular practice of cell culture for PBMC (appropriate density at around 1 million cells per mL).

Solution 2. Overnight culture helps these suspension cells settle down on the bottom.

Solution 3. In order to avoid blowing cells up from the bottom of chamber, it is a good performance to fix one corner for adding reagents and the opposite one for aspirating out reagents.

Solution 4. The procedure of moving the chamber and aspirating or adding any buffer should be as slowly as possible.

Problem 2

No cell clusters are observed in the co-culture of pp65-specific CD4 T cell and pp65-pulsed $\gamma\delta$ T cell (step 3 and 5 in [before you begin](#) and step 17 in [step-by-step method details](#)).

Potential solutions

Solution 1. The main problem is that the cultured CD4 T cells stimulated with pp65 was not successful likely due to the donor cells being unresponsive due to CMV-seronegative status. As mentioned above, the PBMCs that showed clumps within 2 days should be used for subsequent experimental procedures. If not, cells from another donor should be sought.

Solution 2. Failure of activation on $\gamma\delta$ T cell may also lead to little cell cluster in the co-culture system. The control group of co-culture of unactivated $\gamma\delta$ T cell and unstimulated CD4 T cell should show little cell cluster.

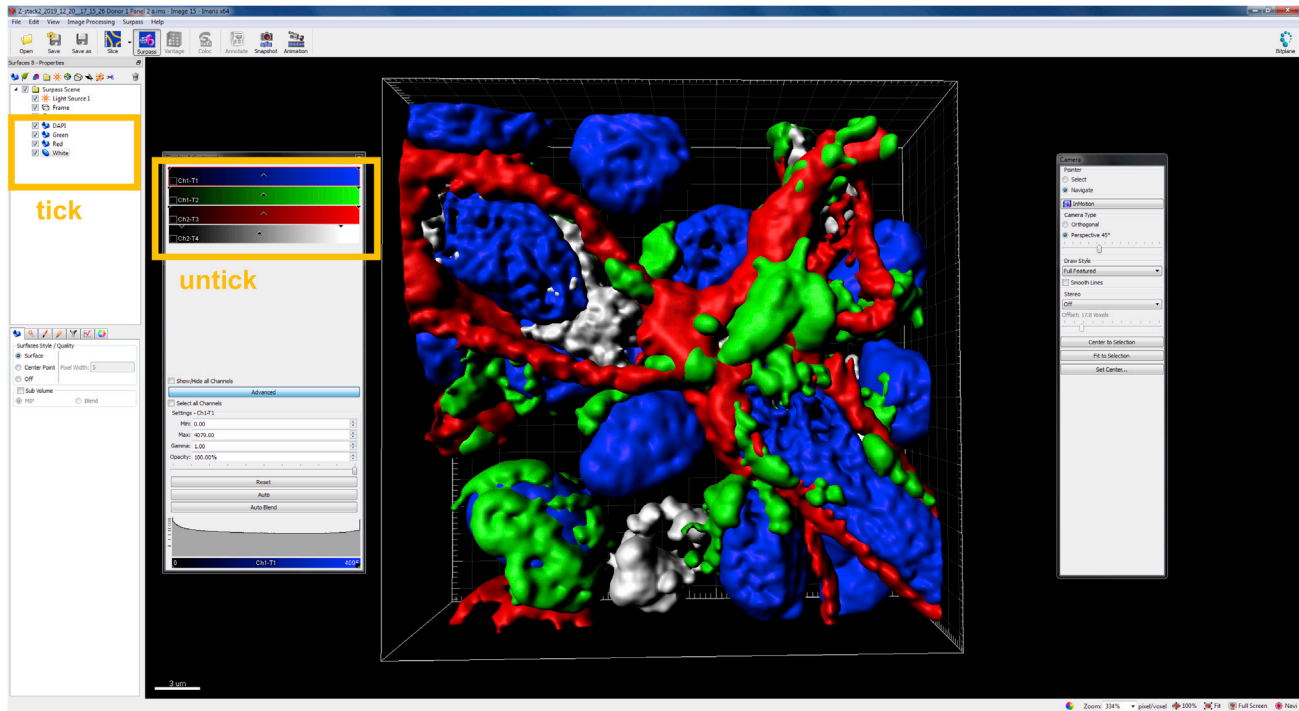


Figure 7. Virtual 3D model for the interface by Imaris

Problem 3

A lot of cell debris shown under the microscope in brightfield (steps 4–15 and 17 in [step-by-step method details](#)).

Potential solutions

Solution 1. Cells might be left dried during experimental procedures of removing the different buffers. To avoid this, after removing the buffer such as PBS, PFA, Perm/Block Buffer and Staining Buffer, the subsequent buffer or solutions should be added into the wells in a timely manner and thoroughly. In our experience, this should be done after removing 3–5 wells.

Solution 2. To avoid cells getting dried in the well-prepared IFA chamber before confocal study, ensure the mounting reagent covers the cells all the time.

Problem 4

Signals are not sufficiently bright for capturing during confocal study (steps 10, 12, and 17 in [step-by-step method details](#)).

Potential solutions

Solution 1. Firstly, a preliminary experiment on testing the incubation concentration of primary antibodies is recommended. This should be based on the recommended range from the manufacturers' instructions to find the optimal concentration for bright signals.

Solution 2. If the tested primary antibody does not work well at the highest concentration, try to increase the concentration of the secondary antibody until the signal is shown clearly.

Problem 5

Noise of images is too high (steps 8, 12, and 17 in [step-by-step method details](#)).

Potential solutions

Solution 1. Firstly, try to adjust the settings of confocal microscope before capturing, for instance, voltage of channels, time of exposure and so on. For instance, increasing laser power is advisable compared to Digital Gain.

Solution 2. If the adjustment on confocal microscope is not helpful, make sure that enough concentration of FBS and Bovine Serum Albumin (BSA) is added into Perm/Block Buffer and the Perm/Block procedure is performed at room temperature (22°C~28°C) in the dark for 20 min.

Solution 3. Reduce the concentration of secondary antibodies to avoid non-specific binding.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Allen Ka Loon Cheung (akcheung@hkbu.edu.hk).

Materials availability

No new materials were generated in this study. Note that the anti- Δ 42PD1 monoclonal antibodies used were described by another publication ([Cheng et al., 2015](#)), which are available upon request.

Data and code availability

No dataset or code was generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.K.L.C. and Z.W.; methodology, A.K.L.C., Y.M., and Z.W.; investigation, A.K.L.C. and Y.M.; writing – original draft, A.K.L.C. and Y.M.; writing – review & editing, A.K.L.C., Y.M., and Z.C.; funding acquisition, A.K.L.C. and Z.C.; supervision, A.K.L.C. and Z.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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