



石蜡切片免疫荧光四标实验报告

1. 实验器材及试剂

1.1 实验器材

名称	厂家	型号
脱水机	DIAPATH	Donatello
包埋机	武汉俊杰电子有限公司	JB-P5
病理切片机	上海徕卡仪器有限公司	RM2016
冻台	武汉俊杰电子有限公司	JB-L5
组织摊片机	浙江金华市科迪仪器设备有限公司	KD-P
烤箱	上海慧泰仪器制造有限公司	DHG-9140A
载玻片	Servicebio	
盖玻片	江苏世泰实验器材有限公司	10212432C
微波炉	格兰仕微波炉电器有限公司	P70D20TL-P4
脱色摇床	Servicebio	TSY-B
涡旋混合器	Servicebio	MX-F
掌上离心机	Servicebio	D1008E
移液枪	Dragon	KE0003087/KA0056573
组化笔	Servicebio	G6100
扫描仪	pannoramic	Pannoramic MIDI:3Dhistech

1.2 主要实验试剂

试剂	厂家	货号	稀释比
无水乙醇	国药集团化学试剂有限公司	100092183	
环保型脱蜡液	武汉赛维尔生物	G1128	
Fitc-TSA	Servicebio	G1222	
CY3-TSA	Servicebio	G1223	
647-TSA	Servicebio	G1224	
柠檬酸 (PH6.0) 抗原修复液	Servicebio	G1202	
EDTA (PH9.0) 抗原修复液	Servicebio	G1203	
EDTA (PH8.0) 抗原修复液	Servicebio	G1206	

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PBS 缓冲液	Servicebio	G0002
自发荧光淬灭剂	Servicebio	G1221
BSA	Servicebio	G5001
3%双氧水	国药集团化学试剂有限公司	
一抗		
荧光二抗	Servicebio	
DAPI	Servicebio	G1012
抗荧光淬灭封片剂	Servicebio	G1401

2. 石蜡切片免疫荧光四标实验步骤

2.1 石蜡切片脱蜡至水: 依次将切片放入环保型脱蜡液 I 10min-环保型脱蜡液 II 10min-环保型脱蜡液 III 10min-无水乙醇 I 5min-无水乙醇 II 5min-无水乙醇 III 5min-蒸馏水洗。

2.2 抗原修复: 组织切片置于盛满 EDTA 抗原修复缓冲液 (PH8.0) 的修复盒中于微波炉内进行抗原修复。中火 8min 停火 8min 转中低火 7min, 此过程中应防止缓冲液过度蒸发, 切勿干片。自然冷却后将玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。(修复液和修复条件根据组织来确定)

2.3 画圈, 双氧水封闭: 切片稍甩干后用组化笔在组织周围画圈 (防止抗体流走), 切片放入 3%双氧水溶液, 室温避光孵育 25 min, 封闭内源性的过氧化物酶, 将玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min

2.4 血清封闭: 滴加 BSA, 封闭 30min。(一抗是山羊来源用 10%兔血清封闭, 一抗其它来源的用 3%BSA 封闭)

2.5 加第一种一抗: 轻轻甩掉封闭液, 在切片上滴加 PBS 按一定比例配好的一抗, 切片平放于湿盒内 4° C 孵育过夜。(湿盒内加少量水防止抗体蒸发)

2.6 加对应的 HRP 标记的二抗: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加与一抗相应种属的 HRP 标记的二抗覆盖组织, 室温孵育 50min。

2.7 加 CY3-TSA: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加 CY3-TSA, 避光室温孵育 10min。孵育完后, 玻片置于 TBST 中在脱色摇床上晃动洗涤 3 次, 每次 5min

2.8 微波处理: 组织切片置于盛满 EDTA 抗原修复缓冲液 (PH8.0) 的修复盒中于微波炉内加热处理, 中火 8min 停火 8min 转中低火 7min, 去掉已经结合到组织上的一抗二抗, 此过程中应防止缓冲液过度蒸发, 切勿干片。

2.9 加第二种一抗: 在切片上滴加 PBS 按一定比例配好的一抗, 切片平放于湿盒内 4° C 孵育过夜。(湿盒内加少量水防止抗体蒸发)

2.10 加对应的 HRP 标记的二抗: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加与一抗相应种属的 HRP 标记的二抗覆盖组织, 避光室温



孵育 50min。

2.11 加 FITC-TSA: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加 TSA, 避光室温孵育 10min。孵育完后, 玻片置于 TBST 中在脱色摇床上晃动洗涤 3 次, 每次 5min

2.12 微波处理: 组织切片置于盛满 EDTA 抗原修复缓冲液 (PH8.0) 的修复盒中于微波炉内加热处理, 中火 8min 停火 8min 转中低火 7min, 去掉已经结合到组织上的一抗二抗, 此过程中应防止缓冲液过度蒸发, 切勿干片。

2.13 加第三种一抗: 在切片上滴加 PBS 按一定比例配好的一抗, 切片平放于湿盒内 4° C 孵育过夜。(湿盒内加少量水防止抗体蒸发)

2.14 加对应的 HRP 标记的二抗: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加与一抗相应种属的二抗覆盖组织, 室温孵育 50min。孵育完后, 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。

2.15 加 647-TSA: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加 TSA, 避光室温孵育 10min。孵育完后, 玻片置于 TBST 中在脱色摇床上晃动洗涤 3 次, 每次 5min

2.16 微波处理: 组织切片置于盛满 EDTA 抗原修复缓冲液 (PH8.0) 的修复盒中于微波炉内加热处理, 中火 8min 停火 8min 转中低火 7min, 去掉已经结合到组织上的一抗二抗, 此过程中应防止缓冲液过度蒸发, 切勿干片。

2.17 加第四种一抗: 在切片上滴加 PBS 按一定比例配好的一抗, 切片平放于湿盒内 4° C 孵育过夜。(湿盒内加少量水防止抗体蒸发)

2.18 加 594 标记的二抗: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后在圈内滴加与一抗相应种属的 594 标记的荧光二抗覆盖组织, 避光室温孵育 50min。孵育完后, 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。

2.19 DAPI 复染细胞核: 切片稍甩干后在圈内滴加 DAPI 染液, 避光室温孵育 10min。

2.20 自发荧光淬灭: 切片稍甩干后, 在圈内加入自发荧光淬灭剂 5min, 流水冲洗 10min。

2.21 封片: 玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。切片稍甩干后用抗荧光淬灭封片剂封片。

2.22 镜检成像: 切片置于扫描仪下采集图像。(DAPI 紫外激发波长 330-380nm, 发射波长 420nm, 发蓝光; FITC 激发波长 465-495nm, 发射波长 515-555 nm, 发绿光; CY3 激发波长 510-560, 发射波长 590nm, 发红光. 647 荧光激发波长 608-648nm, 发射波长 672-712, , 原本为正红色, 为了与 CY3 区分开, 我们设定为粉色光。 594 激发波长 594nm, 发射波长 615nm. 我们设定为紫红色。

3. 技术原理介绍:

主要原理是基于酪胺信号放大 (TSA, Tyramide signal amplification), 以下简称 TSA 技术。TSA 是一种基于辣根过氧化物酶 HRP 的催化活性对靶蛋白或核酸进行高密度原位标记



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的酶学检测方法。技术主要原理为荧光标记的酪胺在 HRP 与 H₂O₂ 的作用下变为活化的酪胺，附着在靶标周围的蛋白酪氨酸残基上。此结合是共价结合，而一抗与靶标、二抗与一抗之间是非共价结合，通过微波修复处理，第一轮的一抗二抗被洗脱，荧光标记的酪胺依然附着在靶标周围。在检测第二个靶标时，相当于全新的一轮标记，无需考虑第二轮的抗体是否与第一轮的抗体产生交叉反应。只需变化不同荧光标记即可实现多个靶标的标记。通过多次重复免疫标记，使用不同的荧光酪胺实现双重或多重荧光染色。

4. 石蜡切片免疫荧光结果判读

DAPI 染出来的细胞核在紫外的激发下为蓝色，阳性表达为相应荧光素标记的红光，粉光，绿光，紫红光。

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Immunofluorescence Protocol (Paraffin-slides)

1. Apparatus and Reagents

1.1 Apparatus

Name	Producer	Model
Dehydrator	DIAPATH	Donatello
Paraffin embedding machine	Wuhan Junjie Electronics Co., Ltd.	JB-P5
pathology slicer	Leica	RM2016
Frozen machine	Wuhan Junjie Electronics Co., Ltd.	JB-L5
Water bath-slide drier	Zhejiang Jinhua Kedi Instrumental Equipment Co., Ltd.	KD-P
Oven	Shanghai Huitai Instrument Manufacturing Co., Ltd.	DHG-9140A
Glass microscope slides	Servicebio	
Coverslips	CITOTEST	10212432C
Microwave	Glanze	P70D20TL-P4
Rocker	Servicebio	TSY-B
Vortex	Servicebio	MX-F
Micro-centrifuge	Servicebio	D1008E
Pipettor	Dragon	KE0003087/KA0056573
Liquid blocker pen	Servicebio	G6100
Slice scanner	Pannoramic	Pannoramic MIDI:3Dhistech

1.2 Major reagents

Name	Producer	Code	Dilution
Ethanol	SCRC	100092183	
Biodewax and Clear Solution	SERVICEBIO	G1128	
EDTA antigen retrieval solution (pH 8.0)	Servicebio	1330-20-7 G1206	
EDTA antigen retrieval solution (pH 9.0)	Servicebio	G1203	

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Sodium citrate antigen	Servicebio	G1202
retrieval solution (pH 6.0)		
PBS solution	Servicebio	G0002
3%H ₂ O ₂	SCRC	
FITC-TSA	Servicebio	G1222
CY3-TSA	Servicebio	G1223
647-TSA	Servicebio	G1224
Spontaneous fluorescence	Servicebio	G1221
quenching reagent		
BSA	Servicebio	G5001
Primary antibody		
Secondary antibody		
DAPI	Servicebio	G1012

2. Procedure

2.1 Deparaffinize and rehydrate: Incubate sections in 3 changes of Biodewax and Clear Solution, 10 min each. Dehydrate in 3 changes of pure ethanol for 5 min. Wash in distilled water.

2.2 Antigen retrieval: Immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min. Be sure to prevent buffer solution evaporate. Let air cooling. Wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Use the right antigen retrieval buffer and heat extent according to tissue characteristics.

2.3 Circle, Block endogenous peroxidase: Wash three times with PBS (pH 7.4) in a Rocker device, 5 min each eliminate obvious liquid, mark the objective tissue with liquid blocker pen. Immerse in 3% H₂O₂ and incubate at room temperature for 15 min, kept in dark place. Then wash again three times with PBS (pH 7.4) in a Rocker device, 5 min each.

2.4 Block with serum: Eliminate obvious liquid, mark the objective tissue with liquid blocker pen. Cover objective tissues with 10% donkey serum (for the case of primary antibody originated from goat) or 3% BSA (for the case of primary antibody originated from others) at room temperature for 30 min.

2.5 First primary antibody: Throw away the blocking solution slightly. Incubate slides with the first primary antibody (diluted with PBS appropriately) overnight at 4 °C, placed in a wet box containing a little water.



2.6 Corresponding secondary antibody marked with HRP: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to first primary antibody in species), incubate at room temperature for 50 min in dark condition.

2.7 CY3-TSA solution: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Incubate slides with CY3-TSA solution (diluted with TBST appropriately) for 10 min in dark condition. After that, wash slides three times with TBST in a Rocker device, 5 min each.

2.8 Microwave treatment: Immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min, to remove the primary antibodies and Secondary antibodies combined with tissue. Be sure to prevent buffer solution evaporate.

2.9 Second primary antibody: Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 °C, placed in a wet box containing a little water.

2.10 Second corresponding secondary antibody marked with HRP: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to second primary antibody in species), incubate at room temperature for 50 min in dark condition.

2.11 FITC-TSA solution: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Incubate slides with FITC-TSA solution (diluted with TBST appropriately) for 10 min in dark condition. After that, wash slides three times with TBST in a Rocker device, 5 min each.

2.12 Microwave treatment: Immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min, to remove the primary antibodies and Secondary antibodies combined with tissue. Be sure to prevent buffer solution evaporate.

2.13 Incubate the third primary antibody: Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 °C, placed in a wet box containing a little water.

2.14 The third corresponding secondary antibody marked with HRP: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to second primary antibody in species), incubate at room temperature for 50 min in dark condition.

2.15 647-TSA solution: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Incubate slides with 647-TSA solution (diluted with TBST appropriately) for 10 min in dark condition. After that, wash slides three times with TBST in a Rocker device, 5 min each.

2.16 Microwave treatment: Immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min, to remove the primary antibodies and Secondary antibodies



combined with tissue. Be sure to prevent buffer solution evaporate.

2.17 Incubate the fourth primary antibody: Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 °C, placed in a wet box containing a little water.

2.18 The fourth corresponding secondary antibody marked with 594 labeled Fluorescence secondary antibody: Wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to second primary antibody in species), incubate at room temperature for 50 min in dark condition.

2.19 DAPI counterstain in nucleus: Incubate with DAPI solution at room temperature for 10 min, kept in dark place.

2.20 Spontaneous fluorescence quenching: Eliminate obvious liquid, incubate slides with spontaneous fluorescence quenching reagent for 5 min, then wash slides under flowing water for 10 min.

2.21 Mount: Wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Throw away liquid slightly, then coverslip with anti-fade mounting medium.

2.22 Detection and collect images by slice scanner: DAPI glows blue by UV excitation wavelength 330-380 nm and emission wavelength 420 nm; FITC glows green by excitation wavelength 465-495 nm and emission wavelength 515-555 nm; CY3 glows red by excitation wavelength 510-560 nm and emission wavelength 590 nm. 647 glows pink by excitation wavelength 608-648 nm and emission wavelength 672-712 nm. (647 was originally red, in order to distinguish it from CY3, we set it to pink light.). 594 glows purplish red by excitation wavelength 594 nm and emission wavelength 615 nm. (594 was originally red, in order to distinguish it from CY3, we set it to purplish red light.)

3. Introduction to Technical Principles

The main principle is based on Tyramide Signal Amplification (TSA), hereinafter referred to as TSA technology. TSA is an enzyme detection method based on the catalytic activity of horseradish peroxidase HRP for high-density in situ labeling of target proteins or nucleic acids. The main principle of the technology is that fluorescent-labeled tyramine becomes activated tyramine under the action of HRP and H₂O₂, which attaches to the protein tyrosine residues around the target. This binding is covalent binding, while the primary antibody is non-covalent binding with the target, and the secondary antibody is non-covalent binding with the primary antibody. Through microwave repair treatment, the first round of primary antibody and secondary antibody are eluted, and fluorescently labeled tyramine is still attached around the target. When detecting the second target, it is equivalent to a completely new round of markers, without considering whether the antibody of the second round cross-reacts with the antibody of the first round. Multiple targets can



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be labeled only by changing different fluorescence markers. Double or multiple fluorescent staining was performed using different fluorescent tyramines by repeated immunolabeling.

4. Results

Nucleus is blue by labeling with DAPI. Positive cells are green, red, pink or purplish red according to the fluorescent labels used.

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