石蜡切片免疫荧光三标实验报告

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	浙江省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	Wanwu	
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	Wanwu	TSY-B
涡旋混合器	Wanwu	MX-F
掌上离心机	Wanwu	D1008E
移液枪	Dragon	KE0003087/KA0056573
组化笔	Wanwu	WG1066-1
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	
二甲苯	上海凌峰化学试剂有限公司	1330-20-7	
Fitc-TSA	Wanwu	G1222	1：1000
CY3-TSA	Wanwu	G1223	1：2000
柠檬酸（ PH6.0)抗原修复液	Wanwu	G1202	
EDTA(PH9.0)抗原修复液	Wanwu	G1203	
EDTA（PH8.0)抗原修复液	Wanwu	G1206	
PBS 缓冲液	Wanwu	G0002	
自发荧光淬灭剂	Wanwu	G1221	

BSA	Wanwu	G5001
3%双氧水	国药集团化学试剂有限公司	
一抗		
荧光二抗	Wanwu	
DAPI	Wanwu	G1012
抗荧光淬灭封片剂	Wanwu	G1401

2. 石蜡切片免疫荧光三标实验步骤

2.1 石蜡切片脱蜡至水：依次将切片放入二甲苯I15min-二甲苯II15min-无水乙醇I5min-无水乙醇II5min-85%酒精 5min-75%酒精 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: 切片稍甩干，滴加 BSA，封闭 30min。（一抗是山羊来源用 10%驴血清封闭，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 CY5 标记的荧光二抗：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 CY5 标记荧光二抗覆盖组织，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。

2.15 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。

2.16 淬灭组织自发荧光：玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。在圈内加入自发荧光淬灭剂 5min，流水冲洗 10min。

2.17 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。

2.18 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.。CY5 激发波长 608-648nm, 发射波长 672-712,，CY5 原本为正红色，为了与 CY3 区分开，我们设定为粉色光。

3. 石蜡切片免疫荧光三标结果判读

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 。

Immunofluorescence protocol (paraffin-slides)

1. Apparatus and reagents

1.1 Apparatus

Name	Producer	Model
Dehydrator	DIAPATH	Donatello
Paraffin embedding machine	武汉俊杰电子有限公司	JB-P5
pathology slicer	Leica	RM2016
Frozen machine	武汉俊杰电子有限公司	JB-L5
Water bath-slide drier	浙江省金华市科迪仪器设备有限公司	KD-P
Oven	上海慧泰仪器制造有限公司	DHG-9140A
Glass microscope slides	Wanwu	
Coverslips	CITOTEST	10212432C
Microwave	Glanze	P70D20TL-P4
Rocker	Wanwu	TSY-B
Vortex	Wanwu	MX-F
Micro-centrifuge	Wanwu	D1008E
Pipettor	Dragon	KE0003087/KA0056573
Liquid blocker pen	Wanwu	WG1066-1
Slice scanner	pannoramic	Pannoramic MIDI:3Dhistech

1.2 Major reagents

Name	Producer	Code	Dilution
Ethanol	SCRC	100092183	
Xylene	上海凌峰化学试剂有限公司	1330-20-7	
EDTA antigen retrieval solution (pH 8.0)	Wanwu	G1206	
EDTA antigen retrieval solution (pH 9.0)	Wanwu	G1203	
Sodium citrate antigen retrieval solution (pH 6.0)	Wanwu	G1202	

PBS solution	Wanwu	G0002
3%H2O2	SCRC	
TSA-FITC	Wanwu	G1222
TSA-CY3	Wanwu	G1223
Spontaneouss fluorescence quenching reagent	Wanwu	G1221
BSA	Wanwu	G5001
primary antibody：		
secondary antibody		
DAPI	Wanwu	G1012

2 Procedure

2.1 Deparaffinize and rehydrate: incubate sections in 2 changes of xylene, 15 min each. Dehydrate in 2 changes of pure ethanol for 5 min, followed by dehydrate in gradient ethanol of 85% and 75% ethanol, respectively, 5 min each. 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% H_2O_2 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 ℃, 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 ℃, 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 ℃, placed in a wet box containing a little water.

2.14 The third corresponding secondary antibody marked with CY5 :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 DAPI counterstain in nucleus:incubate with DAPI solution at room temperature for 10 min, kept in dark place.

2.16 Spontaneouss fluorescence quenching: wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each . eliminate obvious liquid,incubate slides with spontaneouss fluorescence quenching reagent for 5 min,then wash slides under flowing water for 10 min.

2.17 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.

2.18 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. CY5 glows pink by excitation wavelength 608-648nm and emission wavelength 672-712nm.(CY5 was originally red, in order to distinguish it from CY3, we set it to pink light.)

3 Results

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