石蜡切片多色免疫荧光 TSA 实验报告

一、实验原理

酪酰胺信号放大(TSA)技术是一类利用辣根过氧化酶（HRP）对靶蛋白进行标记的酶学检测方法，类似常规免疫组化的 DAB 显色方法 。TSA 技术同样采用 HRP 标记的二抗，同样有对应的“显色”步骤 HRP 催化加入反应体系的酪胺荧光素底物，产生活化荧光底物，活化底物可与抗原上的酪氨酸等残基共价结合，使样品上稳定的共价结合酪胺荧光素。之后用热修复法洗去非共价结合的一抗-二抗-HRP 复合物，重复下一种一抗-hrp 二抗来进行第二轮孵育，如此往复就可实现多重标记。

二、实验器材及试剂

1、实验器材

名称	厂家	型号
脱水机	武汉俊杰电子有限公司	JT-12S
生物组织自动包埋机	武汉俊杰电子有限公司	JB-P5
转轮式切片机	徕卡显微系统上海有限公司	HistoCoreBIOCUT
石蜡包埋机（冷台）	武汉俊杰电子有限公司	JB-L5
组织摊片机	武汉俊杰电子有限公司	JK-5
烤箱	天津市莱玻特瑞仪器设备有限公司	GFL125
盖玻片	江苏汇达医疗器械有限公司	710510
载玻片	海门市神鹰实验仪器厂	188109
微波炉	美的	M1-L213B
脱色摇床	武汉赛维尔生物科技有限公司	SYC-Z100

涡旋仪	武汉赛维尔生物科技有限公司	MX-F2
掌上离心机	武汉赛维尔生物科技有限公司	DS-S 100
移液枪	Dragon	KE003068
组化笔	Gene tech	GT1001
荧光显微镜	NIKON	ECLIPSE C1
3D 扫描仪	3D HISTECH	Pannoramic SCAN

2、主要实验试剂

试剂	厂家	货号
无水乙醇	杭州宏达化工仪器有限公司	SJ003614
二甲苯	国药集团化学试剂有限公司	10023418
EDTA 修复液（PH8.0）	杭州浩克生物技术有限公司	HKI0003
PBS 缓冲液	杭州浩克生物技术有限公司	HK0002
BSA 牛血清白蛋白	杭州浩克生物技术有限公司	HKW2084
一抗：xxxx 红 二抗：HRP 超敏山羊抗兔鼠通用二抗	杭州浩克生物技术有限公司	HKI0029
Flare520 绿	杭州浩克生物技术有限公司	HKI0014
DAPI（即用型）	杭州浩克生物技术有限公司	HKI0005
抗荧光淬灭封片剂	杭州浩克生物技术有限公司	HKI0007-1

三、实验步骤

1. **石蜡切片脱蜡至水：**依次将切片放入二甲苯I 12 min—二甲苯II 12min—无水乙醇I 6min—95%酒精 6 min—85%酒精 6 min—蒸馏水洗。

2. **抗原修复：**组织切片置于盛满 EDTA 修复液（PH8.0）的修复盒中于微波炉内进行抗原修复，中火 8min 停火 8min 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。
3. **阻断内源性过氧化物酶：**切片加上 3%的双氧水，室温孵育 25min，将玻片置 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。
4. **画圈：**用组化专用的组化笔沿着组织外围轮廓画一个与组织间隔 3-4 毫米的小圈，然后加入足量的 PBS 保证后续依次加入的封闭血清，一抗，二抗，以及显色剂能完全覆盖组织，而不沿着玻片流走。
5. **血清封闭：**在组化圈内滴加 3%BSA 均匀覆盖组织，室温封闭 30min 以上。
6. **加一抗：**切片稍甩干后用组化笔在组织周围画圈（防止抗体流走），在圈内滴加按一定比例稀的抗体覆盖组织。切片平放于湿盒内 4℃ 孵育过夜。
7. **加二抗：**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，避光室温孵育 50min。
8. **信号放大：**将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min 。滴加相对应颜色 Flare 信号放大试剂，3-5min.将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。双标或者多标的实验从这一步结束后重复步骤 2-7，标记第二个、或者第三个抗体，最后再进入步骤 8 即可。
9. **DAPI 复染细胞核：**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 DAPI 染液，避光室温孵育 8min。
10. **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。
11. **镜检拍照：**切片于尼康倒置荧光显微镜下观察并采集图像，根据对应波长选择相应通道。

四、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试剂标记的颜色（具体荧光通道参考下表）。

Flare 染料	激发波长	发射波长
DAPI 蓝色	350	420
Flare480 青绿	450	480
Flare520 绿	490	520
Flare570 红	550	570
Flare620 橙	590	620
Flare690 粉	630	690
Flare780 红外	750	780

五、注意事项

1. 注意切片脱蜡是否彻底；
2. 实验过程中切片勿干片；
3. 荧光放大试剂为不可逆，需谨慎添加。

Paraffin sections polychromatic immunofluorescence experiment report (TSA)

1. Experimental principle

Tyramide signal amplification (TSA) technique is a kind of enzymatic detection method using horseradish peroxidase (HRP) to label target protein, which is similar to the conventional immunohistochemical DAB color development method. TSA technology also uses HRP labeled secondary antibodies, and also has corresponding "color rendering" steps to catalyze the addition of tyramine fluorescein substrate in the reaction system to produce an activated fluorescent substrate, which can covalently bind tyramine fluorescein and other residues on the antigen to make the sample covalently bind tyramine fluorescein. After that, the non-covalently bound primary - secondary -HRP complex was washed by thermal repair method, and the next primary -hrp secondary - antibody was repeated for the second round of incubation, so that multiple labeling could be achieved repeatedly.

2. Laboratory equipment and reagents

2.1 Laboratory equipment

Equipment	Manufacturers	Model
Dehydrator	Wuhan Junjie Electronics Co., Ltd	JT-12S
Paraffin embedding machine	Wuhan Junjie Electronics Co., Ltd	JB-P5
Rotary microtome	Shanghai Leica Instrument Co., Ltd	HistoCoreBIOCUT
Frozen table	Wuhan Junjie Electronics Co., Ltd	JB-L5

Tissue machine	Wuhan Junjie Electronics Co., Ltd	JK-5
Oven	Tianjin Leibo Terry Equipment Co., Ltd	GFL125
Coverslips	Jiangsu Huida Medical Instruments Co., Ltd	710510
Glass microscope slides	Haimen Shenying Experimental Equipment Factory	188109
Microwave oven	Midea	M1-L213B
Rocker	Servicebio	SYC-Z100
Vortex	Servicebio	MX-F2
Micro-centrifuge	Servicebio	DS-S 100
Pipettor	Dragon	KE003068
Liquid Blocker PAP Pen	Gene tech	GT1001
Fluorescence microscope	NIKON	ECLIPSE C1
3D Imaging System	3D HISTECH	Pannoramic SCAN

2.2 Laboratory reagents

Reagents	**Manufacturers**	**Catlog**
Anhydrous ethanol	Hangzhou Hongda Chemical Instrument Co., Ltd	SJ003614
Xylene	Sinopharm Chemical Reagent Co., Ltd	10023418
EDTA Antigen repair solution（PH8.0）	Hangzhou Haoke Biotechnology Co., Ltd	HKI0003
PBS solution	Hangzhou Haoke Biotechnology Co., Ltd	HK0002

BSA	Hangzhou Haoke Biotechnology Co., Ltd	HKW2084
Primary antibody：xxxx		
Second antibody：HRP hypersensitive goat anti-rabbit secondary antibody	Hangzhou Haoke Biotechnology Co., Ltd	HKI0029
Flare520	Hangzhou Haoke Biotechnology Co., Ltd	HKI0014
DAPI	Hangzhou Haoke Biotechnology Co., Ltd	HKI0005
Anti-fluorescence quenching sealing tablets	Hangzhou Haoke Biotechnology Co., Ltd	HKI0007-1

3. Experimental procedure

3.1 Dewaxing and hydration: Put the sections into xylene I 12 min - xylene II 12 min - anhydrous ethanol I 6 min - 95% Ethyl alcohol for 6 min - 85% Ethyl alcohol for 6 min, rinsing with distilled water.

3.2 Repair: The tissue sections were placed in a repair box filled with EDTA repair solution (PH8.0) and the antigen repair was carried out in a microwave oven. The repair procedure was: medium fire for 8min, ceasefire for 8min, and medium low fire for 7min. During this process, excessive evaporation of buffer solution should be prevented and the pieces should not be dried. After natural cooling, the slide was placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time.

3.3 Block endogenous peroxidase： The sections were incubated with 3% hydrogen peroxide at room temperature for 25min. The slides were placed in PBS (PH7.4) and washed three times on a decolorizing shaker for 5min each time.

3.4 Draw a circle around the tissue: Use a histochemical pen to draw a small circle 3-4 mm apart from the tissue along the outer outline of the tissue, and then add enough PBS to ensure that the subsequent addition of blocking serum, primary antibody, secondary

antibody, and color development agent can completely cover the tissue without flowing along the sections.

3.5 Serum blocking: After the section is slightly dried, draw a circle around the tissue with a histochemical pen (to prevent the antibody from flowing away), and add 3%BSA to the circle and seal it at room temperature for more than 30 minutes.

3.6 Add primary antibody: After the section is slightly dried, draw a circle around the tissue with a tissue pen (to prevent the antibody from flowing away), and add a certain proportion of dilute antibody to cover the tissue in the circle. The slices were incubated flat in a wet box at 4℃ overnight.

3.7 Add secondary antibody: The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time. After the slices were slightly dried, the tissue covered by the second antibody of the corresponding species of the first antibody was added to the ring and incubated at room temperature for 50min away from light.

3.8 TSA: The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time. Add the corresponding color Flare signal amplification reagent, 3-5min. Place the slide in PBS (PH7.4), shake and wash it on the decolorizing shaker for 3 times, 5min each time. After this step, repeat steps 2-7, label the second or third antibody, and then proceed to step 8.

3.9 DAPI restaining nuclei: The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing table for 3 times, 5 min each time. Blot the excess PBS in the ring and then add DAPI dye solution, and incubate at room temperature for 8min away from light.

3.10 Sealing: The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing table for 3 times, 5min each time. Remove the slices and seal them with anti-fluorescence quenching tablets.

3.11 Microscopy photography: The sections were observed and the images were collected under a fluorescence microscope, and the corresponding channels were selected according to the corresponding wavelength.

4. Interpretation of results

The nuclei stained by DAPI are blue under ultraviolet excitation, and the positive expression is the color labeled by the corresponding fluorescent reagent (refer to the table below for specific fluorescence channels).

Flare Dye	Excitation wavelength	Emission wavelength
DAPI	350	420
Flare480	450	480
Flare520	490	520
Flare570	550	570
Flare620	590	620
Flare690	630	690
Flare780	750	780

5. Precautions

5.1 Pay attention to whether the slice dewaxing is thorough;

5.2 Don't let the slices dry out during the experiment;

5.3 The addition of fluorescence amplification reagent is irreversible and should be added with caution.