冰冻切片 IFtunel+IF 双标实验报告

1.实验器材及试剂

1.1 实验器材

名称	厂家	型号
冰冻切片机	Thermo	Cryotome E
载玻片	wanwu	
盖玻片	江苏世泰实验器材有限公司	10212432C
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
冰箱	青岛海尔股份有限公司	BCD-192TGN
正置荧光显微镜	日本尼康	NIKON ECLIPSE C1
成像系统	日本尼康	NIKON DS-U3

1.2 主要实验试剂

试剂	厂家	货号
4%多聚甲醛	wanwu	G1101
PBS 缓冲液	wanwu	G0002
蛋白酶 K	wanwu	G1205
破膜液	wanwu	G1204
BSA	wanwu	G5001
Tunel 试剂盒	wanwu	G1501
一抗		
荧光二抗		
DAPI	wanwu	G1012
抗荧光淬灭封片剂	wanwu	G1401

2.冰冻切片荧光 tunel+免疫荧光双标实验步骤

2.1 冰冻切片固定：冰冻切片 37℃烘箱烘烤 10-20min，控干水分。置于 4%多聚甲醛固定 30min，于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。

2.2 蛋白酶 K 修复：切片稍甩干后用组化笔在组织周围画圈（防止液体流走），在圈内滴加蛋白酶 K 工作液覆盖组织，37 度温箱孵育 22min。将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。(蛋白酶 K 工作液配置方法，原液：PBS=1:9)

2.3 破膜：切片稍甩干后在圈内滴加破膜工作液覆盖组织，常温下孵育 20min，将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。(破膜液为 0.1%triton. 配置方法，triron 原液：PBS=1:1000)

2.4 室温平衡：切片稍甩干后在圈内滴加 buffer 覆盖组织，buffer 常温孵育 10min.

2.5 加反应液：按片子数量和组织大小取 tunel 试剂盒内适量 TDT 酶 ,dUTP，buffer 按 1:5:50 比例混合，加到圈内覆盖组织，切片平放于湿盒内，37℃恒温箱孵育 2 小时，湿盒内加少量水保持湿度。

2.6BSA 封闭:将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min，在圈内滴加用 3%BSA 均匀覆盖组织，室温封闭 30min。

2.7 加一抗：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℃ 孵育过夜。（湿盒内加少量水防止抗体蒸发）

2.8 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。

2.9DAPI 复染细胞核：切片用 PBS（PH7.4）洗涤 3 次，每次 5min。去除 PBS 后在圈内滴加 DAPI 染液，避光室温孵育 10min。

2.10 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。

2.11 镜检拍照：切片于荧光显微镜下观察并采集图像。（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）。

三．冰冻切片荧光 tunel+免疫荧光双标结果判读

DAPI 染出来的细胞核在紫外的激发下为蓝色，TUNEL 试剂盒为 FITC 荧光素标记，阳性凋亡细胞核为绿色，一抗标记的阳性细胞为红色

Tunel & immunofluorescence double staining protocol (frozen-slides)

1. Apparatus and reagents

1.1 Apparatus

Name	Producer	Model
Freezing microtome	Thermo	Cryotome E
Glass microscope slides	wanwu	
Coverslips	CITOTEST	10212432C
Rocker	wanwu	TSY-B
Vortex	wanwu	MX-F
Micro-centrifuge	wanwu	D1008E
Pipettor	Dragon	KE0003087/KA0056573
Liquid blocker pen	wanwu	WG1066-1
Refrigerator	Haier	BCD-192TGN
Ortho-Fluorescent Microscopy	Nikon	NIKON ECLIPSE C1
Imaging system	Nikon	NIKON DS-U3

1.2 Major reagents

Name	Producer	Model
4% **paraformaldehyde**	wanwu	G1101
PBS solution	wanwu	G0002
Proteinase K	wanwu	G1205
Permeabilize solution	wanwu	G1204
BSA	wanwu	G5001
Tunel assay kit		G1501
Primary antibody		
Fluorescent-labelled secondary anyibody		
DAPI	wanwu	G1012
anti-fade mounting medium	wanwu	G1401

2.Procedure

2.1Fix frozen-slides: restore frozen-slides to room temperature and dry in air. Fix in cold acetone 10 min and then dry in air. Wash three times with PBS (pH 7.4) in a Rocker device, 5 min each.
2.2 Antigen retrieval: eliminate obvious liquid, mark the objective tissue with liquid blocker pen. Add proteinase K working solution to cover objectives and incubate at 37℃ for 25 min. then wash three times with PBS (pH 7.4) in a Rocker device, 5 min each.（Method for configuring working solution of proteinase K, stock solution: PBS=1:9）

2.3 Permeabilization: eliminate excess liquid, add permeabilize working solution to cover objective tissue, then incubate at room temperature for 20 min. wash three times with PBS (pH 7.4) in a Rocker device, 5 min each.（The membrane breaking fluid is 0.1% triton. Configuration method, triron stock solution: PBS=1:1000）

2.4 Equilibrium at room temperature: After the slices are slightly dried, buffer is added to the tissues in the circle, and the buffer is incubated at room temperature for 10 minutes
2.5 Tunel reaction: Take appropriate amount of TDT enzyme, dUTP and buffer in the tunel kit according to the number of slices and tissue size and mix at 1:5:50 ratio。 Prepare this reaction solution according to demand before use. Add this mixture to objective tissue placed in a flat wet box, incubate at 37℃ for 2 h. be sure to keep the wet box moist by adding water.
2.6 BSA blocking: wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Add 3% BSA to cover the marked tissue to block non-specific binding for 30 min BSA.
2.7 Primary antibody: throw away the blocking solution slightly. Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 ℃, placed in a wet box containing a little water.
2.8 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 primary antibody in species), incubate at room temperature for 50 min in dark condition.
2.9 DAPI counterstain in nucleus: wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then incubate with DAPI solution at room temperature for 10 min, kept in dark place.
2.10 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.
2.11 Microscopy detection and collect images by Fluorescent Microscopy. 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.

3.Results

The nuclei stained by DAPI are blue under the excitation of ultraviolet light, the tunel kit is labeled with FITC fluorescein, the positive apoptotic cells are labeled with green light, and the primary antibody is labeled with red.