Three Color Staining of Spleen Sections

As an example, to stain for $CD4^+$ T cells, $\lambda 1^+$ B cells and PNA^+ B cells use the following reagents:

1. α-CD4 (GK1.5, Goat α-Rat Ig-AP_1)
2. α-λ1-biotin (LS136-biotin)
3. PNA-HRP

Staining

Throughout this procedure do not use any solutions that contain azide.

All dilutions are made with PBS/5% BSA + 0.1% Tween 20.

All washes are in PBS/5% BSA + 0.1% Tween 20 with shaking unless noted.

All development of colors are done in the dark.

1. Allow sections to come to room temperature.
2. Apply PAP pen (Research Products) around spleen section and allow to dry. This prevents Ab dilutions from floating all over the slide.
3. Place slides in PBS for 20 minutes.
4. Place slides in 0.3% H_2O_2 (diluted in water) for 5 minutes to destroy endogenous peroxidase activity.
5. Wash slides in PBS only for 20 minutes.
6. Incubate slides in PBS/BSA/Tween 20 for 20 minutes.
7. Add 100 µl of α-CD4 + α-λ1-biotin + PNA-HRP. Incubate 1 hr.
8. Wash 3 times, 10 minutes each with shaking.
9. Add 100µl of goat α-rat Ig-AP_1 and incubate 1 hr.
10. Wash 3 times, 10 minutes each wash with shaking.
11. Develop AP_1 with Fast Red (Sigma kit Cat# F-4523).
12. Wash one time with PBS for 10 minutes.
13. Develop HRP with 3-AEC.
14. Wash one time in PBS for 10 minutes.
15. Treat with 1 M HCl for 15 minutes to destroy enzyme activity.

16. Wash two times in PBS, 10 minutes each wash.

17. Add Streptavidin-AP_2 for 1 hr.

18. Wash 3 times, 10 minutes each wash.

19. Develop AP_2 with Fast BB Blue.

Results: $CD4^+$ T cells are pink, $\lambda 1^+$ B cells are blue and germinal centers B cells are red.