Objectives: To identify proteoglycans (PGs) and collagen fibrils within human dentin by means of a dual immunofluorescent labeling technique under confocal laser scanning microscopy (CLSM) and to investigate the monomer infiltration of two etch-and-rinse adhesives to TPCK-treated trypsin (TRY)-pretreated dentin. Materials and Methods: Thirty-micrometer sections of mid coronal dentin were obtained and etched with 37% phosphoric acid gel for 15 s. After TRY digestion (controls not digested), the sections were subjected to dual immunofluorescent labeling and observed with CLSM to identify the effective removal of PGs. Demineralized dentin matrices treated with TRY and non-treated controls were observed with field emission scanning electron microscopy. Furthermore, two two-step etch-and-rinse adhesives, Adper Single Bond 2 (SB, 3M ESPE) and Prime & Bond NT (PBNT, Dentsply), were applied to the dentin surfaces and the thicknesses of the formed hybrid layers (HL) were evaluated using confocal micro-Raman spectroscopy. Data were analyzed with two-way ANOVA. Results: PGs were localized in the lumen of the dentinal tubules and in peritubular dentin, while the collagen fibrils were localized in intertubular dentin and peritubular dentin. After TRY digestion, the red fluorescence decreased or disappeared, the organic filaments in the lumen of the dentinal tubules disappeared, the tubules were enlarged, and the hybrid layer thickness for adhesives were significantly increased (p<0.001 for both SB and PBNT). No differences (p>0.05) were observed in the thickness of hybrid layers between the adhesives tested. Conclusions: The dual immunofluorescence labeling methodology can be used to study the human dentin matrix without decalcifying the entire dentin fragment. PGs were localized in the lumen of the dentinal tubules and in peritubular dentin, which could depress the infiltration of the adhesive resin monomers. Clinical Significance: Pretreatment of dentin with TRY digestion may be considered as an alternative to achieve optimal dentin bonding results with two-step etch-and-rinse adhesives.