FITC-Conjugated Cyclic RGD Peptides as Fluorescent Probes for Staining Integrin $\alpha_v\beta_3/\alpha_v\beta_5$ in Tumor Tissues

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RUNNING TITLE: FITC-Labeled Dimeric Cyclic RGD Peptides as Fluorescent Probes

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Protocols for Cellular Staining

Cellular Staining of Acetone-Fixed Cells. Tumor cells (U87MG, MDA-MB-435, A549, HT29 and PC-3) were cultured in Lab-Tek 8-well glass chamber slides (BD, Bedford, MA) for >24 h before being used for cellular staining studies. The tumor cells were fixed with -20 °C pre-cold acetone for 15 min. The cells were incubated with 1% BSA in 0.01 M phosphate buffer solution (PBS, pH 7.4) for 30 min at room temperature to block non-specific binding. In overlay experiments, tumor cells were incubated for 1 h at room temperature with 10 μM FITC-conjugated RGD peptides (FITC-RGD_2, FITC-3P-RGD_2 or FITC-Galacto-RGD_2) and anti-human integrin $\alpha_v\beta_3$ antibody (sc-7312, 2.0 μg/mL, BD Biosciences, San Jose, CA). After washing with PBS three times, the tumor cells were incubated with Cy3-conjugated goat anti-mouse antibodies (1:100, V/V, Jackson Immuno-Research Inc., West Grove, PA) for another hour at room temperature. After washing with PBS buffer, the slides were mounted with Dapi Fluormount G (SouthernBiotech, Birmingham, AL) and cover glass. Fluorescence was visualized with an Olympic BX51 fluorescence microscope (Olympus America Inc., Center Valley, PA). Images were acquired using a Hamamatsu digital CCD camera ORCA-R2 (Hamamatsu Photonics K.K., Japan) with Olympus MetaMorph software. All pictures were taken under 400 × magnifications with the same exposure time. Brightness and contrast adjustments were made equally to all images. The overlays were made using Olympus MetaMorph software.

Cellular Staining of Living Cells. Tumor cells (U87MG, MDA-MB-435, A549, HT29 and PC-3) were rinsed with ice-cold PBS for 5 min, and then incubated with 1% BSA in PBS for 30 min on ice to block non-specific binding. Tumor cells were incubated with 10 μM FITC-labeled cyclic RGD peptides (FITC-RGD_2, FITC-3P-RGD_2 or FITC-Galacto-RGD_2) for 2 h on ice. After washing with PBS buffer, tumor cells were analyzed for fluorescent intensity. In overlay experiments, tumor cells were incubated with 1% BSA in PBS for 30 min on ice, and then incubated for 2 h on ice with 10 μM FITC-conjugated cyclic RGD peptide and anti-human integrin $\alpha_v\beta_3$ antibody (sc-7312: 2.0 μg/mL). After washing with PBS buffer, tumor cells were then incubated for another 1 h on ice with Cy3-conjugated goat anti-mouse antibodies (1:100, V/V). In the blocking experiments, U87MG glioma cells were incubated for 2 h with 10 μM FITC-Galacto-RGD_2 in the presence of 1 mM $c(RGDfK)_2$. FITC-3P-RGK_2 (10 μM) was used as negative control to demonstrate RGD specificity of cyclic RGD peptides for integrin $\alpha_v\beta_3$.

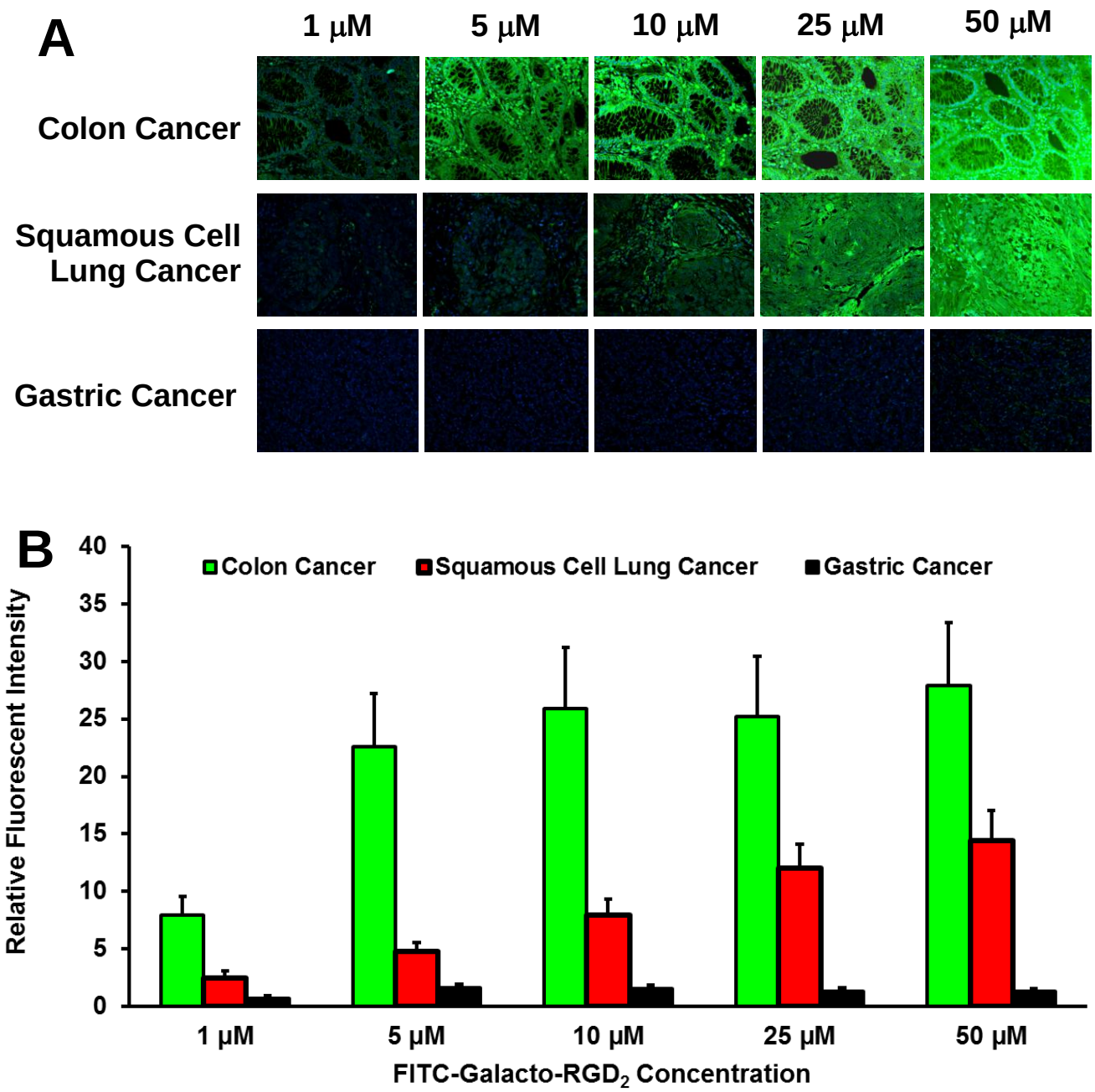

Figure SI1. **A**: Selected microscopic images (Magnification: 200 ×) of colon cancer, squamous cell lung cancer and gastric cancer tissues stained with FITC-Galacto-RGD_2 (1, 5, 10, 25 and 50 μM). **B**: Quantification of fluorescence intensity on tumor slices to illustrate the concentration-dependence of tumor fluorescence intensity. The relative integrin $\alpha_v\beta_3/\alpha_v\beta_5$ density was quantified as the percentage of the area positively stained with FITC-Galacto-RGD_2 over the total area of each image.

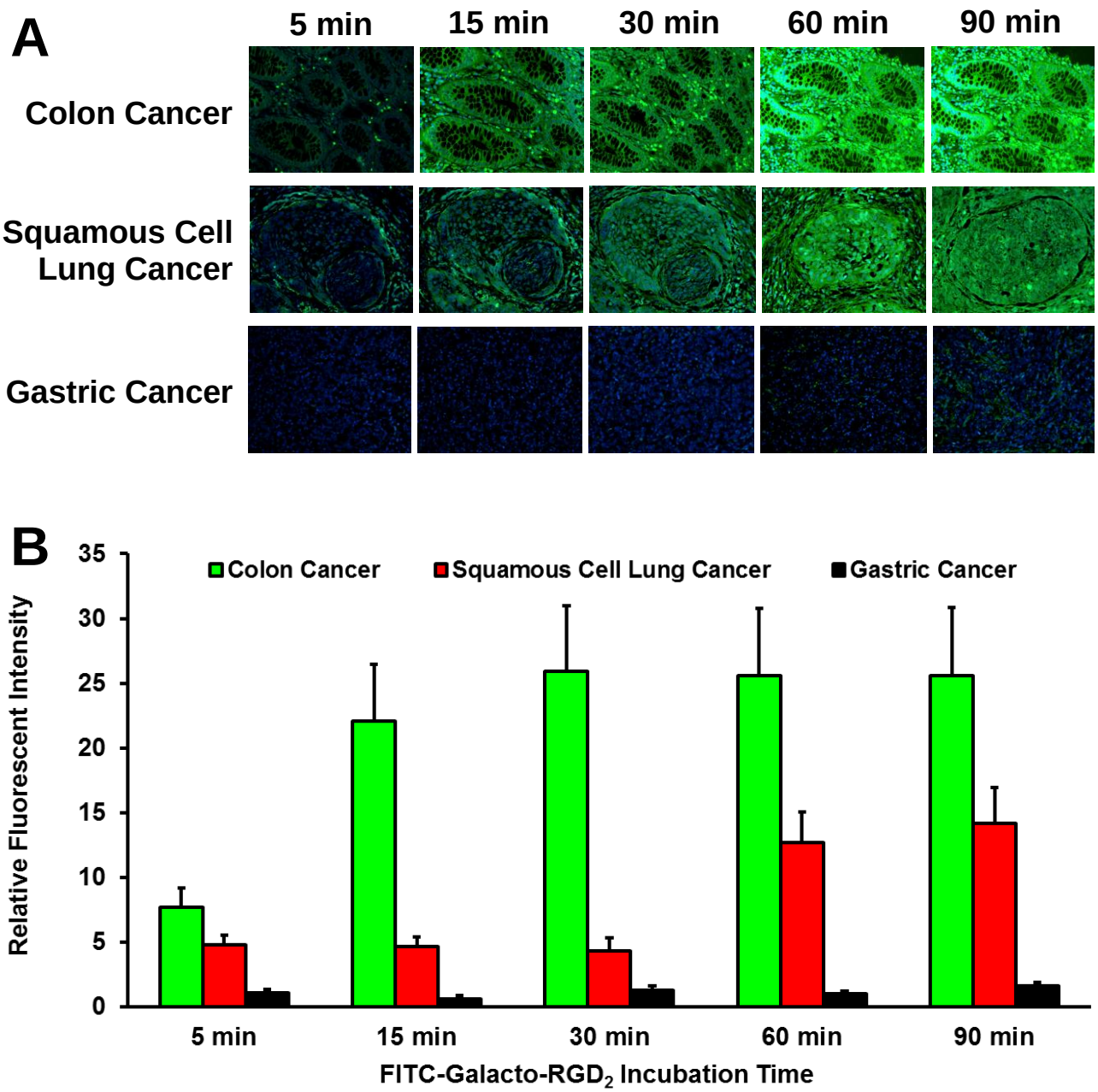

Figure SI2. **A**: Microscopic images (Magnification: 200×) of colon cancer, lung squamous cell cancer and gastric cancer tissues stained with 50 μM FITC-Galacto-RGD_2. B: The tumor tissue staining kinetics as illustrated by the fluorescence intensity at different incubation times (5 – 90 min) to illustrate the dependence of tumor fluorescence intensity on incubation time. The relative integrin $\alpha_v\beta_3/\alpha_v\beta_5$ density was quantified as the percentage of the area positively stained with FITC-Galacto-RGD_2 over the total area of each image.

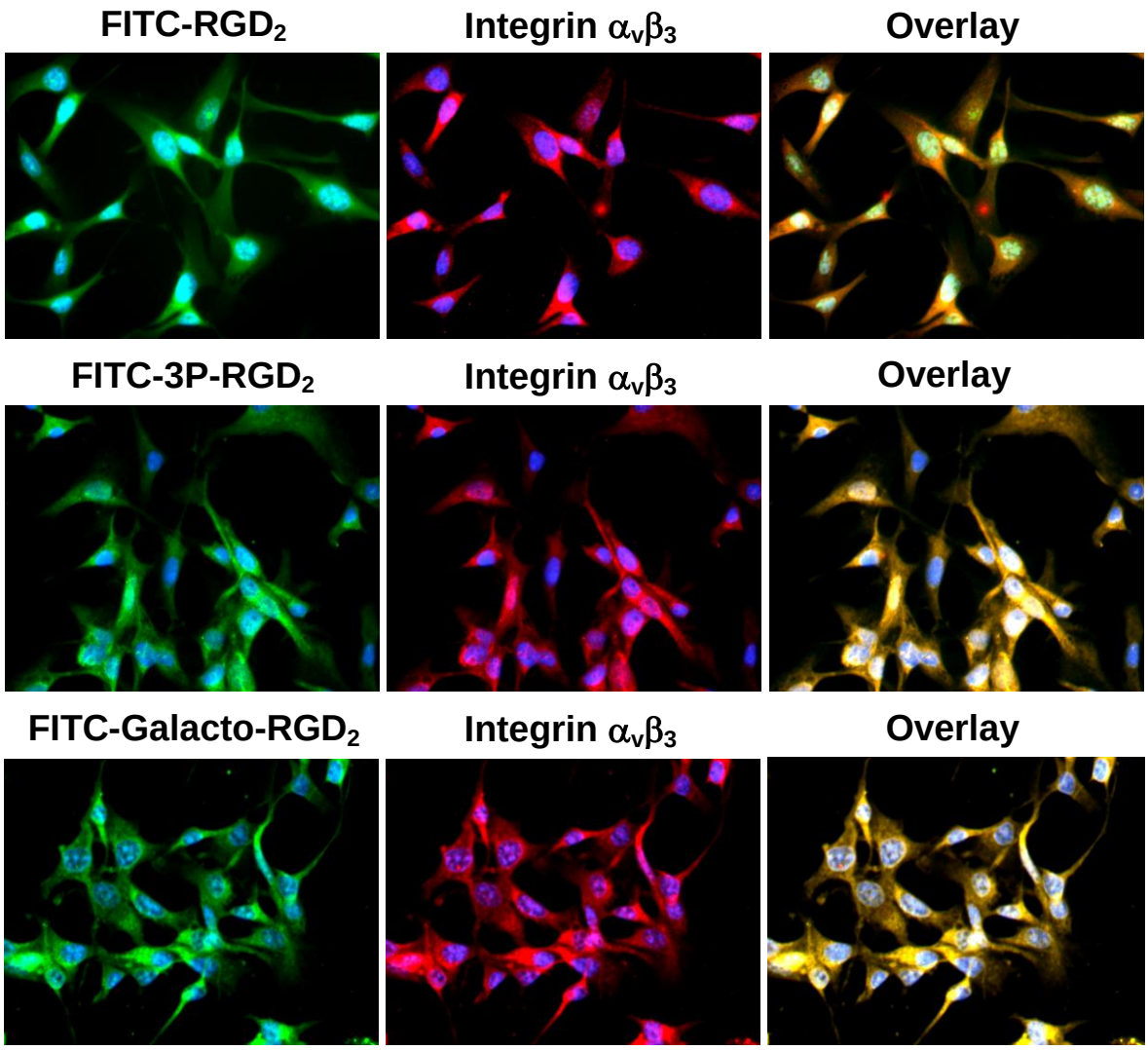

Figure SI3. Selected microscopic images (Magnification: 400×) of U87MG glioma cells stained with FITC-conjugated cyclic RGD peptides (Green) and mouse anti-human integrin $\alpha_v\beta_3$ antibody (sc-7312) detected with Cy3 conjugated goat anti-mouse antibody (red). Blue color indicates the presence of nuclei stained with DAPI. In the overlay images, orange and yellow colors indicate co-localization of FITC-labeled cyclic RGD peptides (Top: FITC-RGD_2; Middle: FITC-3P-RGD_2; Bottom: FITC-Galacto-RGD_2) with sc-7312. Co-incubation of FITC-RGD_2, FITC-3P-RGD_2 or FITC-Galacto-RGD_2 with anti-integrin $\alpha_v\beta_3$ antibody resulted in positive staining of U87MG glioma cells. The fluorescence intensity on tumor cells stained with FITC-RGD_2 was slightly lower than that stained with FITC-3P-RGD_2 and FITC-Galacto-RGD_2, due to its lower integrin $\alpha_v\beta_3/\alpha_v\beta_5$ binding affinity. The fluorescence signals distributed throughout the entire U87MG tumor cells. Nuclear localization was also observed for FITC-RGD_2, FITC-3P-RGD_2 and FITC-Galacto-RGD_2. The orange and yellow colors in the overlay images clearly indicated the co-localization of FITC-conjugated cyclic RGD peptides and anti-integrin $\alpha_v\beta_3$ antibody. FITC-3P-RGD_2 and FITC-Galacto-RGD_2 were useful as fluorescent probes for integrin $\alpha_v\beta_3/\alpha_v\beta_5$ staining of U87MG cells.

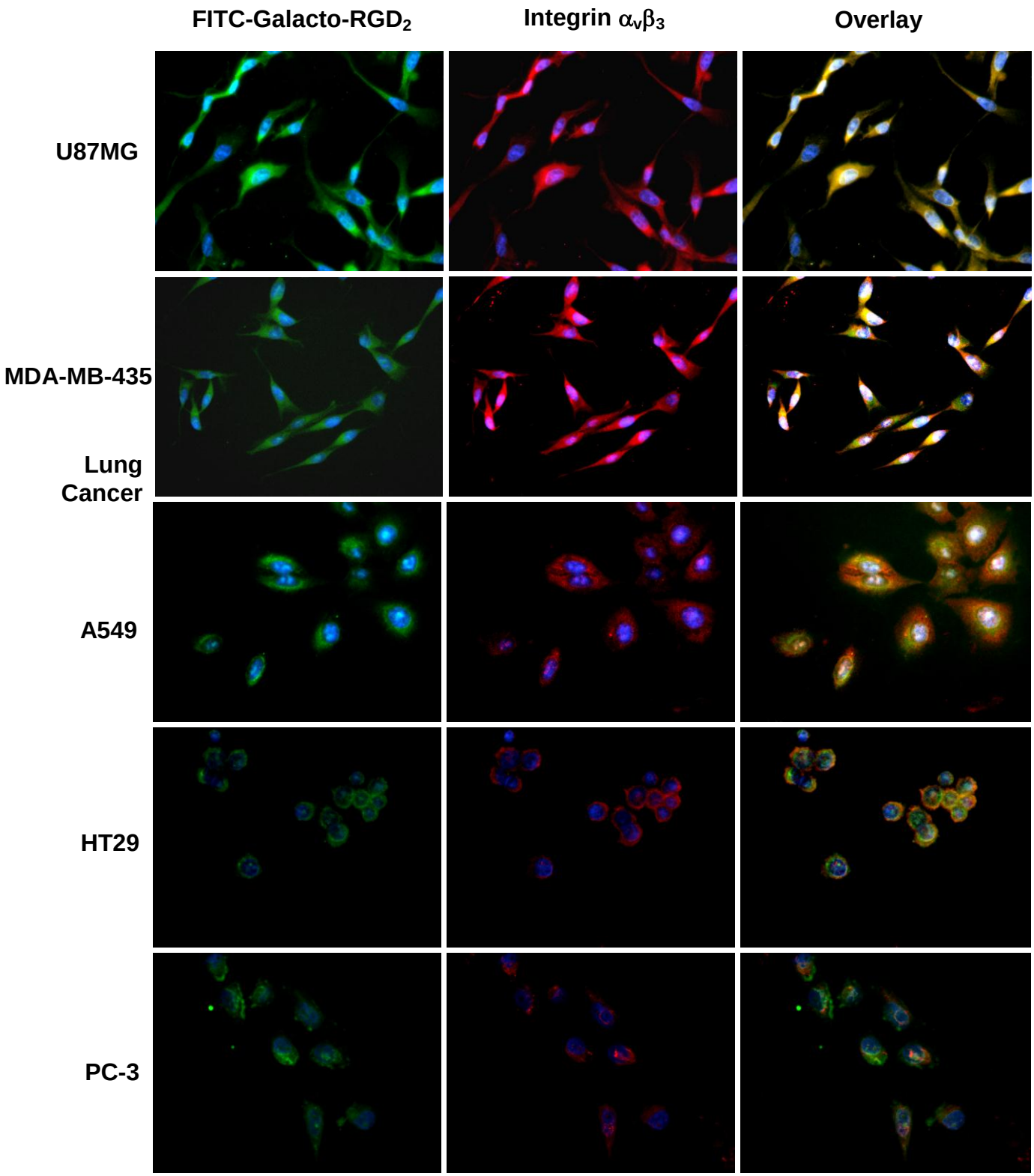

Figure SI4. Microscopic images (Magnification: 400×) of acetone-fixed human cancer cells stained with FITC-Galacto-RGD_2 (green) and mouse anti-human $\alpha_v\beta_3$ monoclonal antibody (sc-7312) detected with Cy3-conjugated goat anti-mouse antibody (red). Blue color indicates the nuclei stained with DAPI. Orange or yellow in overlay image indicates co-localization of FITC-Galacto-RGD_2 (green) and anti-human integrin $\alpha_v\beta_3$ monoclonal antibody (sc-7312).

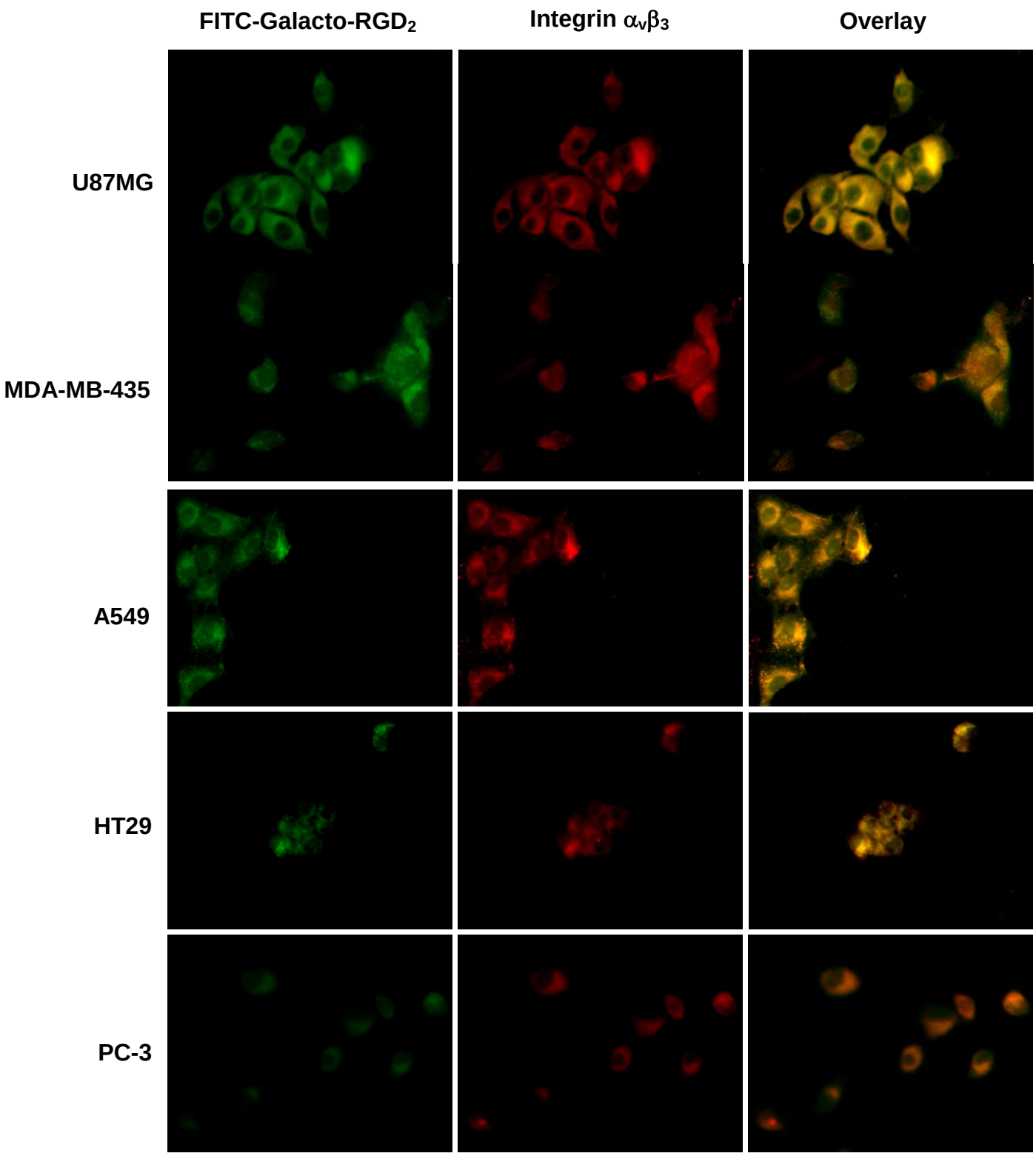

Figure SI5. Microscopic images (Magnification: 400×) of living tumor cells stained with FITC-Galacto-RGD_2 (green) and mouse anti-human $\alpha_v\beta_3$ monoclonal antibody (sc-7312) detected with Cy3-conjugated goat anti-mouse antibody (red). Orange and yellow in overlay images indicate co-localization of FITC-Galacto-RGD_2 (green) and anti-human $\alpha_v\beta_3$ monoclonal antibody (sc-7312) in living tumor cells.