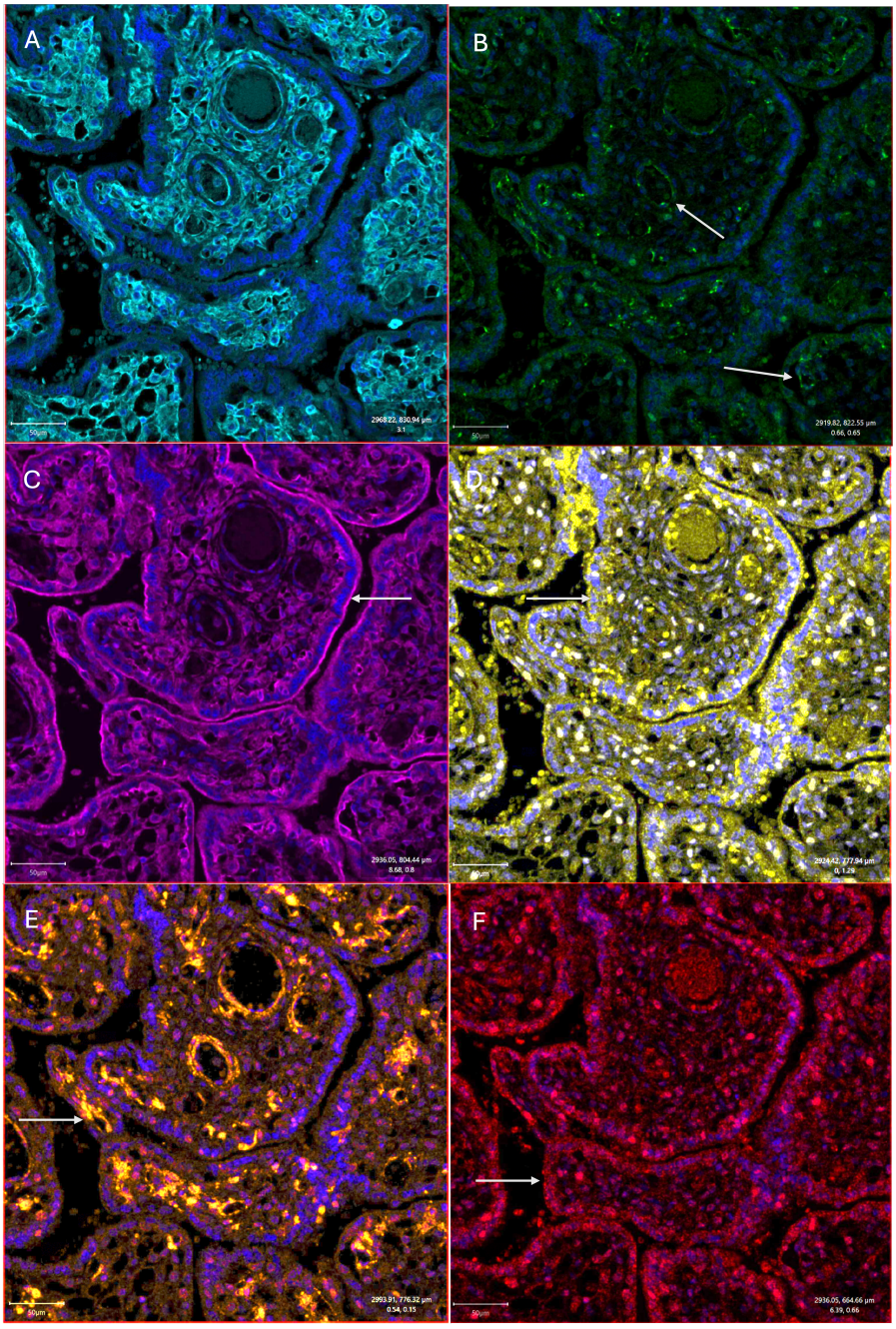

Figure 4.8: **Multiplex IF on Human Placenta**

Staining for; **A)** HLA (cyan) - Diffuse cytoplasmic and membranous staining is seen in this human placenta tissue **B)** Glut1 (green) - Arrows point to tubular structures consistent with endothelial cells lining blood vessels **C)** EGFR (purple)- arrows indicate membranous staining in trophoblast cells **D)** p-EGFR (yellow) - arrows indicate cytoplasmic staining in trophoblasts **E)** FAK (orange) - arrows indicate cytoplasmic staining in trophoblasts and **F)** p-FAK (red)- arrows indicate cytoplasmic staining in trophoblasts. All sections counter stained with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI) (Blue). Scale bars = 50 μm

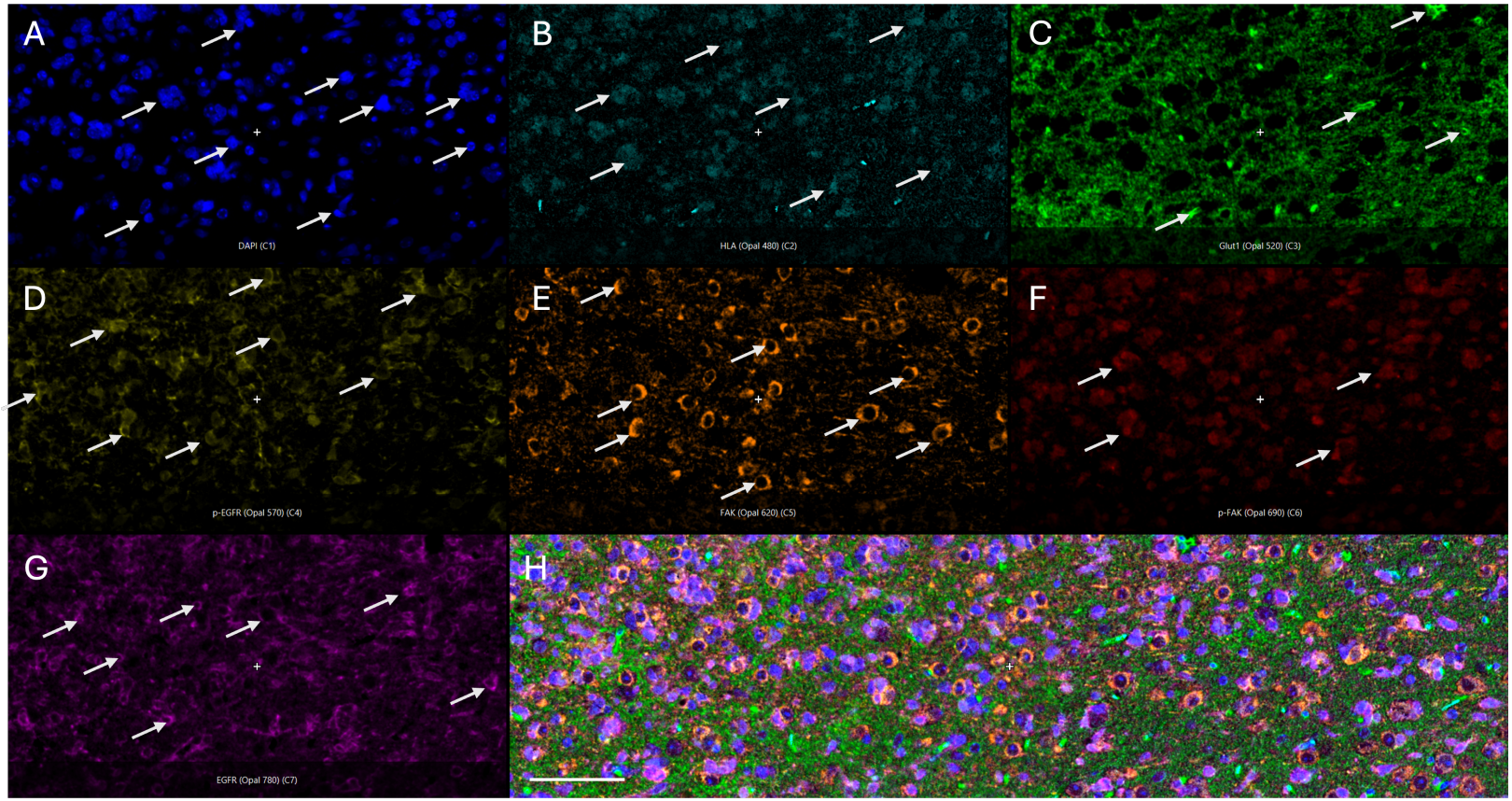

Figure 4.9: **Multiplex IF of Study Sections**

Staining of human GBM in mouse brain xenograft for; **A)** nuclear stain 4,6-diamidino-2-phenylindole (DAPI) (Blue) **B)** HLA (cyan) - Arrows point to positive staining for human cells **C)** Glut1 (green) - Arrows point to tubular structures consistent with endothelial cells lining blood vessels **D)** p-EGFR (yellow) **E)** FAK (orange) **F)** p-FAK (red) **G)** EGFR (purple) and **H)** Overlay of all stains. Arrows indicate examples of positive staining. Scale bars = 50 μm

4.2.4 Segmentation of Cells and Blood Vessels

To clarify how the spatial organisation of EGFR and FAK signalling operates in GBM cells relative to stiffer structures, such as blood vessels, within the generally softer brain parenchyma, the open source software QuPath's Cell Detection Tool was utilised. This tool distinguishes cells from the surrounding ECM using the DAPI staining channel. A pixel threshold approach determines positive DAPI staining to identify cell nuclei. Using this detection, cell boundaries are approximately delineated via expansion. The parameters applied were as follows:

Parameter	**Settings**
Requested Pixel Size	0.5 μm
Background Radius	8 μm
Use Opening By Reconstruction	Yes
Median Filter Radius	0 μm
Sigma	1.5 μm
Minimum Area	10 μm^2
Maximum Area	250 μm^2
Threshold	2
Split by Shape	Yes
Cell Expansion	2 μm
Include Cell Nucleus	Yes

Table 4.2: **QuPath Settings for Cell Detection**

The cells were then classified as mouse cells or glioblastoma cells using the QuPath (open source) object classifier based on HLA staining in the cytoplasm of detected cells. The object classifier was trained using a deep learning model using an Artificial Neural Network with a manually selected sample set of $>$500 glioblastoma cells and $>$500 mouse cells from each of the xenograft sections. The same object classifier was saved and used

to classify cells for each of the xenograft sections. This generalised solution for cell segmentation and classification worked well for large datasets of whole slide images of the four xenograft assays used.

The blood vessels were successfully segmented from the rest of the ECM using GLUT-1 staining and a simple pixel threshold method with the following parameters:

Parameter	**Settings**
Resolution	Full (0.5μm/px)
Prefilter	Maximum (dilation)
Smoothing Sigma	0
Threshold	4
Sigma	1.5 μm
Minimum Area	10 μm^2
Maximum Area	250 μm^2
Threshold	2
Split by Shape	Yes
Cell Expansion	2 μm
Include Cell Nucleus	Yes

Table 4.3: **QuPath Settings for Blood Vessel Segmentation**

4.2.5 Classification of GBM Cells Migrating Individually or in a Cluster

GBM cells were further classified into individual cells or those that appeared as a cluster to determine whether the mode of migration influences invasion patterns. A Delaunay triangulation analysis, which is a geometric technique used to create a mesh of triangles from a set of points in a plane, was used to perform nearest-neighbour analysis. The key property of Delaunay triangulation is that no point in the set lies inside the circumcircle of any triangle in the triangulation. (Gabriel and Sokal [1969], Goltsev et al. [2018]).