

Quantitation of Macropinocytosis in Glioblastoma Based on High-Content Analysis

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Research article

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Abstract

Background: Macropinocytosis serves as an internalization pathway for extracellular fluid, albumin and dissolved molecules. Assessing macropinocytosis has been challenging in the past because manual acquisition in combination with visual evaluation of images is laborious, making it difficult for high-throughput applications. So, there is a need to develop sensitive and specific methods.

Methods: This paper proposed a quantitative and time-saving method for macropinocytosis detection based on high-content analysis (HCA). Meanwhile, cell proliferation was tested by means of CCK8.

Results: The term “macropinosome index” was defined to estimate macropinocytosis and allow comparison between different cell lines and treatments. Furthermore, we demonstrated that macropinocytosis can promote Glioblastoma (GBM) cells survival in glutamine deficient conditions which resemble tumor microenvironment. **Conclusions:** HCA represents a novel, non-subjective and high-throughput assay for macropinocytosis assessment. Besides, Gln deprivation increased the macropinosome index in GBM cells, which points to the possible exploitation of this process in the design of GBM therapies.

Background

Macropinocytosis is a type of endocytosis that involves non-selectively uptake extracellular fluid via plasma membrane ruffling into large intracellular vesicles known as macropinosomes. Macropinosomes can be visualized on the basis of the ability of cells to internalize extracellular medium containing tetramethylrhodamine labeled high-molecular-mass dextran (TMR-dextran), an established marker of macropino-

cytosis. Macropinocytosis functions in cancer cells as an important nutrient-scavenging pathway by which extracellular proteins move to lysosomes for degradation [1].

It was reported that intratumoral inhibition of macropinocytosis decreases amino acid levels [2]. Furthermore, macropinocytosis promotes both tumor growth and chemotherapy resistance in vivo by uptake necrotic cell debris [3, 4]. The level of micropinocytosis can increase in the context of oncogenic transformation (e.g., oncogenic RAS or PI3K) or tumor suppressor mutations (e.g., loss of PTEN) [5-6]. Besides, enhanced macropinocytosis has also been found by the upregulation of growth factors [7].

Recent works have described the tumor can adapt to Glutamine (Gln) deprivation [8], implying the presence of unknown regulatory mechanisms that enable tumor progression. A full revealing of how macropinocytosis is regulated would be of great significance in understanding how the tumor proliferates in the microenvironment of nutrient deprivation, ultimately leading to new therapeutic approaches.

In this context, this study aimed to verify in vitro whether Gln deprivation can change the extent of macropinocytosis in GBM cells, which normally have moderate levels of macropinocytosis, in addition to

proposing a new method to develop a high-throughput assay.

Methods

Preparing Gln deprivation medium

Gln deprivation medium was prepared to contain inorganic salts, i.p. 4.5 g/L glucose, 2.5 g/L NaHCO₃, 0.11 g/L C₃H₃NaO₃, and Phenol Red, according to the composition of DMEM.

Seeding cells

The special 96 well plate should be irradiated by ultraviolet in biological safety cabinet for at least 12 h before seeding cells. Pipette 100 µl cells into 96 well black plates using a multichannel pipette or a single channel pipette. Incubate cells in complete fresh medium for 24 h in an incubator (37 °C, 5% CO₂) to ensure attachment.

Labeling macropinosomes

The visualization of macropinosomes in cells is based on the ability of cells to internalize extracellular fluid containing fluorescently tagged TMR-dextran (Thermo Fisher Scientific, D1818), which is universally used to mark macropinosomes. After starvation for 24h, the former medium was replaced with complete medium including 75 µM TMR-dextran. Incubate the cells in a cell culture incubator (37 °C, 5% CO₂) for 30 min.

Labeling cytoplasm

After complete rinsing of the sample with pre-cooling PBS, fix the sample in 4% methanol-free paraformaldehyde solution in pre-cooling PBS for 15 min at room temperature. Wash the sample three times with pre-cooling PBS, 5 min at one time. Permeabilize the sample in 0.5% Triton™ X-100 in PBS for 15 min. Incubate the sample for 40 min in 50 µl phalloidin (Thermo Fisher Scientific, A12379) staining solution for each well at room temperature.

Labeling nuclei

Incubate the sample in the 50 µl DAPI (Abcam, ab104139) for each well for 15 min at room temperature. Wash the sample three times with pre-cooling PBS, 5 min at one time. Add one drop of anti-fluorescence quencher (BBI, E675011) for each well. Then observe at once by high content or store in the dark at 2-6°C and observe within 12 h.

Capturing images

A High-content screening (PerkinElmer Operetta CLS) equipped for phase contrast with a standard optical filter set including DAPI, Alexa Fluor 488 and Alexa 568 was used. Select random fields of one well to test

and set an appropriate exposure time to be applied to all the samples for each individual channel being analyzed. Capture images using the ×63 water-immersion objective and the Harmony software platform. The FITC and Alexa 568 exposure times range from 200 to 500 ms. Then select all the wells containing cells. To ensure the reliability of results, obtain 50-100 random fields for every well.

Data analysis

After capturing, the data will be saved automatically. Then load the data in measurement. Set script parameters including contrast, area and the detection algorithm to properly segment nuclei, cytoplasm and spots in every cell. Finally, add “Define Result”, define Variable a as “Number of Spots”, define Variable b as “Cell Area”, and define Formula as “a/b”, then click Apply or Save to use next time.

CCK8 test

Incubate cells in the complete fresh medium for 24 h in an incubator (37 °C, 5% CO₂) to ensure attachment of cells. The cells were then washed with PBS, and complete fresh medium was initiated by replacement with the Gln-deprived medium. Add 10 µl Cell Counting Kit 8 (APE BIO, k1018) solution to each well. Protect from the light and incubate for 2 h at 37 °C, then measure the absorbance at 450 nm as results of day 0. Similarly, repeat the steps every 24 h to get the results from day 0 to 5.

Statistical analysis

Data are expressed as mean±s.e.m. and analyzed by Student’s t-test with significance defined as P<0.05.

Results

Macropinocytosis detection was established based on high-content analysis (HCA).

Schematic workflow of the protocol as shown in Fig. 1. For simplicity, we multiply macropinosome index values by 10. We came up with macropinosome index to measure the level of macropinocytosis. The macropinosome index values for cancer cells <0.5 and >1 are defined as low macropinocytosis cells and highly macropinocytosis cells, respectively.

As an example of the implementation of this protocol, we assessed the extent of macropinocytosis in GBM cells. Sample images from the various steps of quantification are shown in Fig.2. Nuclei and cytoplasm are labeled with DAPI and phalloidin, respectively. U251 was maintained in serum-free media and treated with vehicle or 75 µM EIPA for 30 min, after which spots representing macropinosomes were labeled by uptake of TMR-dextran. After merged images capturing, the data will be saved automatically. Then load the data in measurement. Set script parameters including contrast, area and the detection algorithm to properly segment nuclei, cytoplasm and spots in every cell. The total spots number and total cell area are then recorded and tabulated as shown in Table 1. EIPA treatment significantly inhibited macropinosome index compare to control group. The mean macropinosome index of EIPA treatment and control group is 1.16±0.10 and 0.43±0.19 (P<0.001), respectively.

GBM survive in the microenvironment of the Gln deprivation

The apoptosis level increased and the morphology became longer for GBM cells in the microenvironment of Gln deprivation (Fig.3, A). We found that GBM cells grew slower in the absence of Gln, demonstrating that Gln starvation hindered proliferation to different extents. However, GBM cells can survive for at least five days in such situation (Fig.3, B and C).

Gln deprivation promote macropinocytosis in GBM

To determine whether stimulated macropinocytosis is a feature of GBM exposing to nutrient stress, we compared TMR-dextran uptake of U251 and U87 cell lines in Gln deprivation medium and complete medium by the described protocol (Fig.4, A). Gln deprivation increased the macropinosome index in GBM cells (Fig.4, B and C).

Discussion

Macropinocytosis is a kind of pathway for internalization involving vesicles 50-300 nm in diameter. In the analysis of micropinocytosis, the concept of micropinocytosis index was defined by Lee SW et al [9], which is widely applied, especially in the field of cancer research [10-12]. Generally, macropinocytosis assay mainly includes two discontinuous stages: (1) images capture using fluorescent microscope; (2) image quantification by means of ImageJ software. Furthermore, the second stage also contains several steps, such as count nuclei, adjust the brightness, subtract the background, apply smooth function, adjust threshold, convert the image to a binary image and use the Analyze Particles function. Obviously, the assay is complicated and time-consuming because fluorescence microscopy and analysis cannot be performed simultaneously. Besides, it is necessary to wash coverslips and seed cells on the coverslips to capture images.

In this study, we developed a quantitative image analysis protocol for macropinocytosis based on HCA. The special capability of HCA is a spot counting feature that allows the determination of the quantity and size of macropinosomes in each cell. The extent of macropinocytosis in GBM cells was quickly and accurately estimated by means of this protocol.

Glutamine (Gln) is considered a conditionally essential amino acid for tumor cells. Breakdown of Gln through glutaminolysis gives rise to glutamate (Glu), a critical precursor of most other nonessential amino acids [13]. As tumors continue to grow, increased nutrient demand and poor vascularization lead to Gln deprivation in the microenvironment. Glioma cells are able to release amounts of Glu, which potentially promotes tumorigenesis through activation of ionotropic receptors [14]. Gln addiction has been proposed as a mark of glioblastoma (GBM) [15], which means that Gln deprivation reduces proliferation and tumorigenicity. Our results are consistent with previous studies. However, We also found that GBM cells can survive for at least five days in such situation.

Tumor cells can survive Gln deprivation by activating cell cycle arrest genes [16], performing metabolic reprogramming [17], or sustaining purine availability by Gln synthetase[18], but other mechanisms can also contribute to cell survival. Gln deprivation-induced scavenging mechanism has received considerable attention in macropinocytosis [19]. This is an endocytic process that supports proliferation of tumor cells in the medium devoid of essential amino acids [20]. Macropinocytosis promotes survival and reduces chemosensitivity in GBM cells [21, 22]. We identified macropinocytosis as a mechanism by which GBM cells adapt nutritionally deficient microenvironment. Thus, macropinocytosis may represent a predictive biomarker for cancer therapy.

Conclusions

In this study, we developed a novel assay for macropinocytosis based on HCA. This assay enables simultaneous analysis of spots, nuclei and cytoplasm in a wide variety of cellular models, representing a novel, non-subjective and high-throughput assay for macropinocytosis assessment. Besides, we demonstrated that Gln deprivation increased the macropinosome index in GBM cells, which points to the possible exploitation of this process in the design of GBM therapies.

Abbreviations

GBM: glioblastoma

HCA: high-content analysis

Gln: Glutamine

TMR-dextran: tetramethylrhodamine labeled high-molecular-mass dextran

Declarations

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Author contributions

Study Design: YW Pan, GQ Yuan. Study Implementation: B Wang, X Yao, Q Dong. Data Analysis and Interpretation: B Wang, XF Wang, H Yin, Q Li, XQ Wang, Y Liu. Initial Manuscript Draft: B Wang. Final Review and Approval: GQ Yuan. All authors have read and approved the manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Conflict of interest

All the authors declared that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

The data of macropinosome index values are available in this published article. Other data generated or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1. Sample quantification of macropinosome index in U251 treated with EIPA.

Treatment	Field no.	Total spots no.	Total cell Area (μm^2)	Macropinosome index ($\times 10$)	Mean \pm s.e.m.
Con	1	1723	15150.8	1.14	1.16 \pm 0.10 ^{***}
	2	2623	22589.1	1.16	
	3	1395	10660.7	1.31	
	4	1790	17793.8	1.01	
	5	1100	8692.32	1.26	
	6	813	7088.14	1.15	
	7	1672	15391.9	1.09	
EIPA	1	78	8424	0.09	0.43 \pm 0.19
	2	153	6159.18	0.25	
	3	208	4100.61	0.51	
	4	319	6923.86	0.46	
	5	175	2872.1	0.61	
	6	105	1900.38	0.55	
	7	477	8423.5	0.57	

Note: ***, P<0.001.

Figures

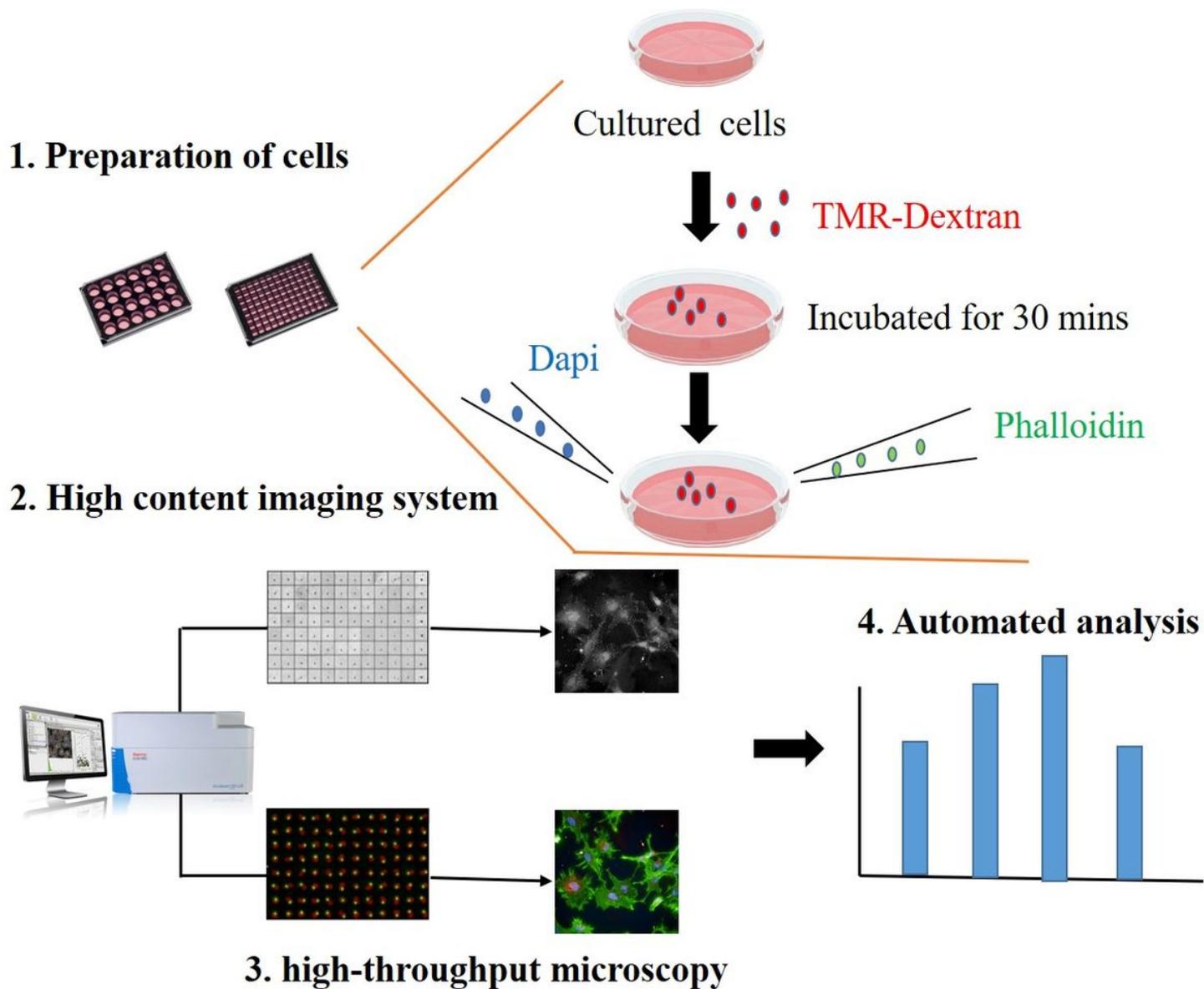


Figure 1

Schematic workflow of the protocol as shown in Fig. 1.

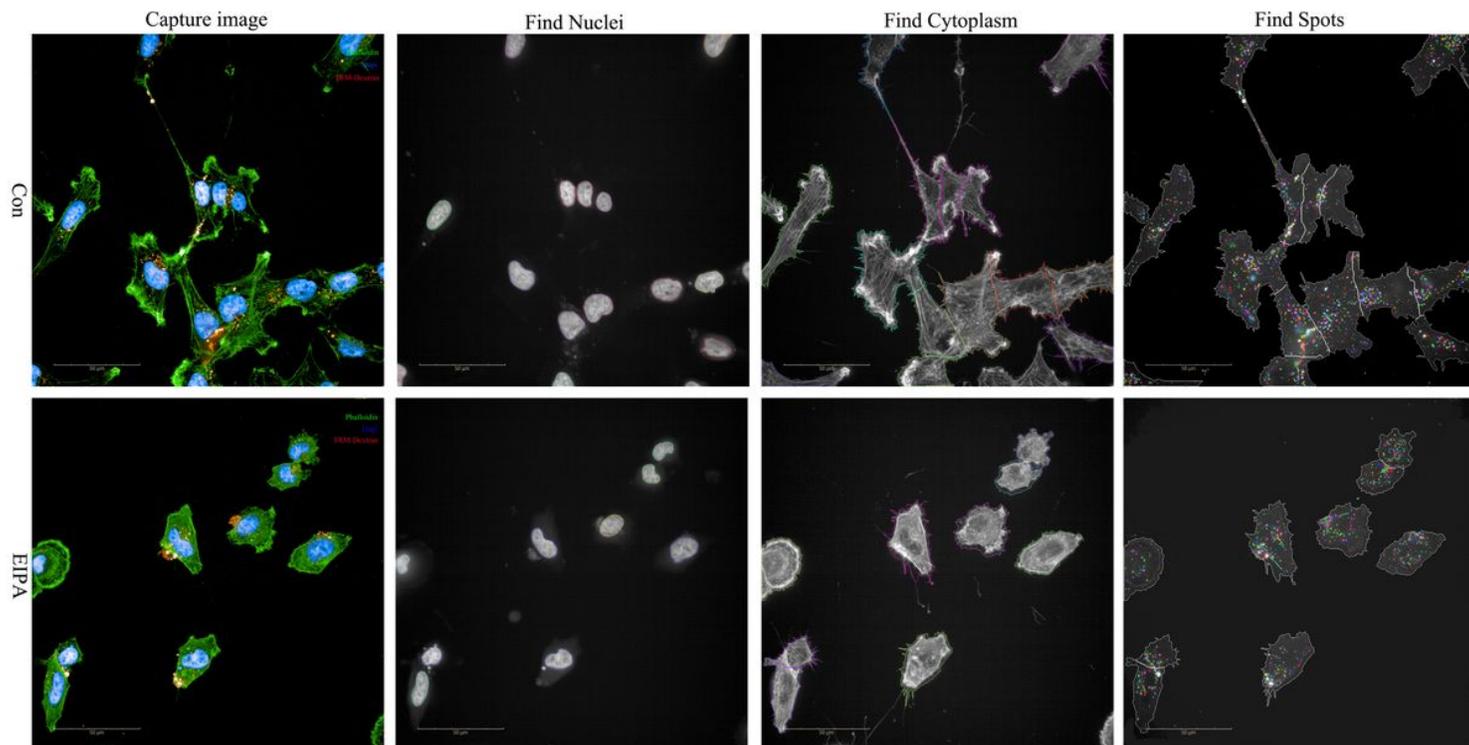


Figure 2

As an example of the implementation of this protocol, we assessed the extent of macropinocytosis in GBM cells. Sample images from the various steps of quantification are shown in Fig.2.

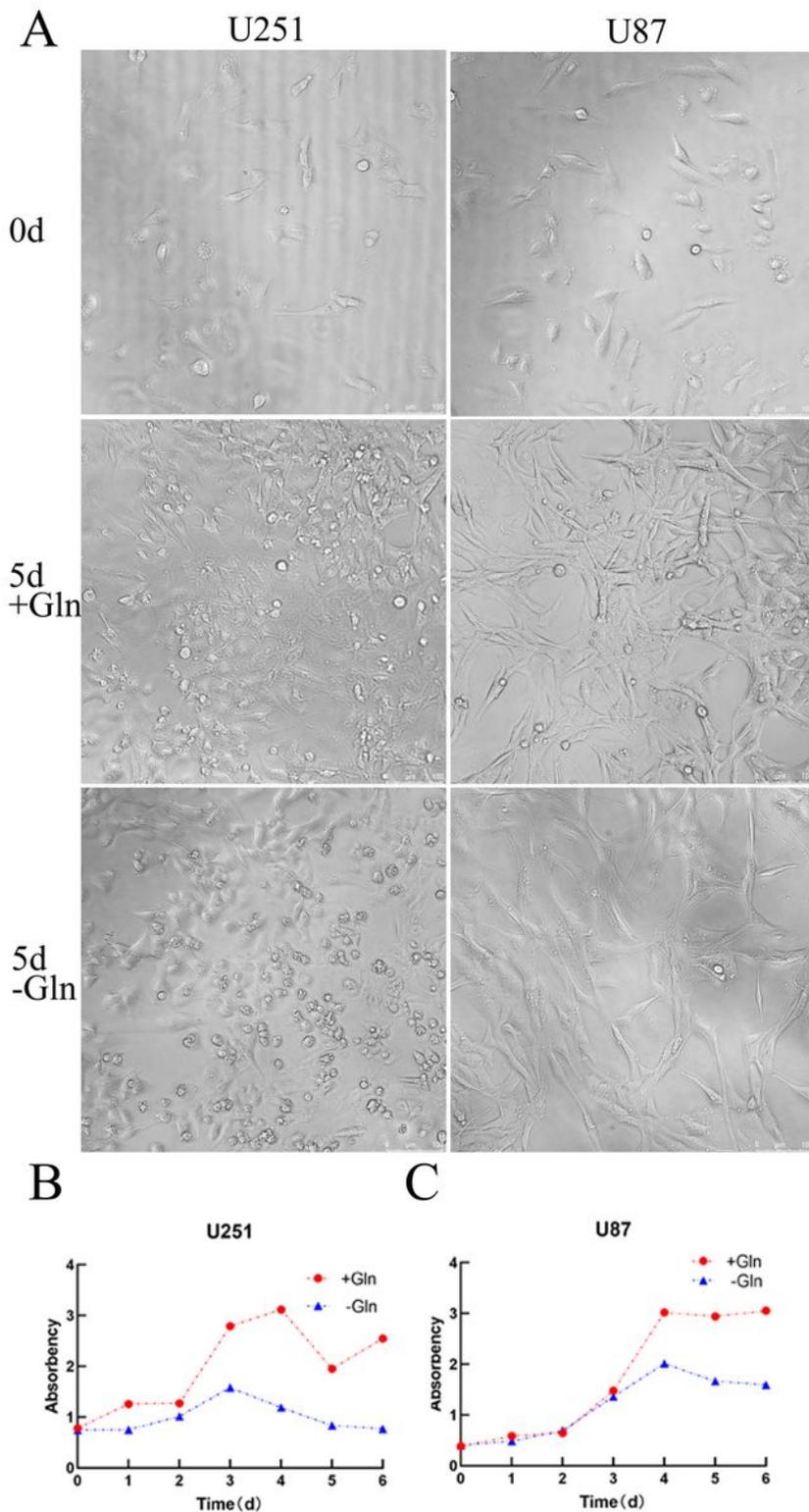


Figure 3

The apoptosis level increased and the morphology became longer for GBM cells in the microenvironment of Gln deprivation (Fig.3, A). We found that GBM cells grew slower in the absence of Gln, demonstrating that Gln starvation hindered proliferation to different extents. However, GBM cells can survive for at least five days in such situation (Fig.3, B and C).

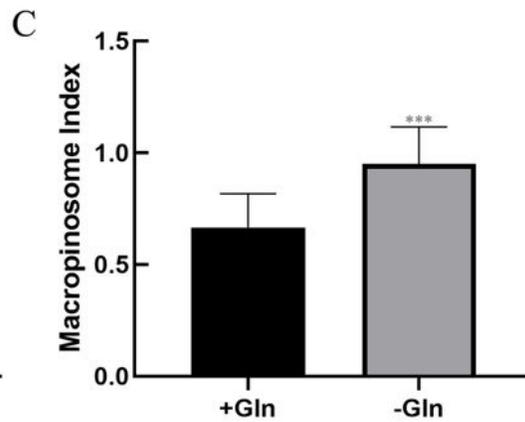
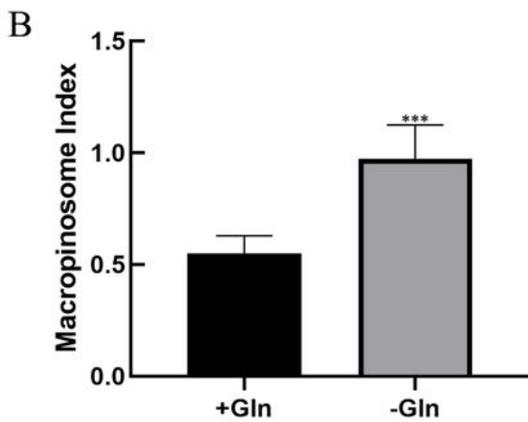
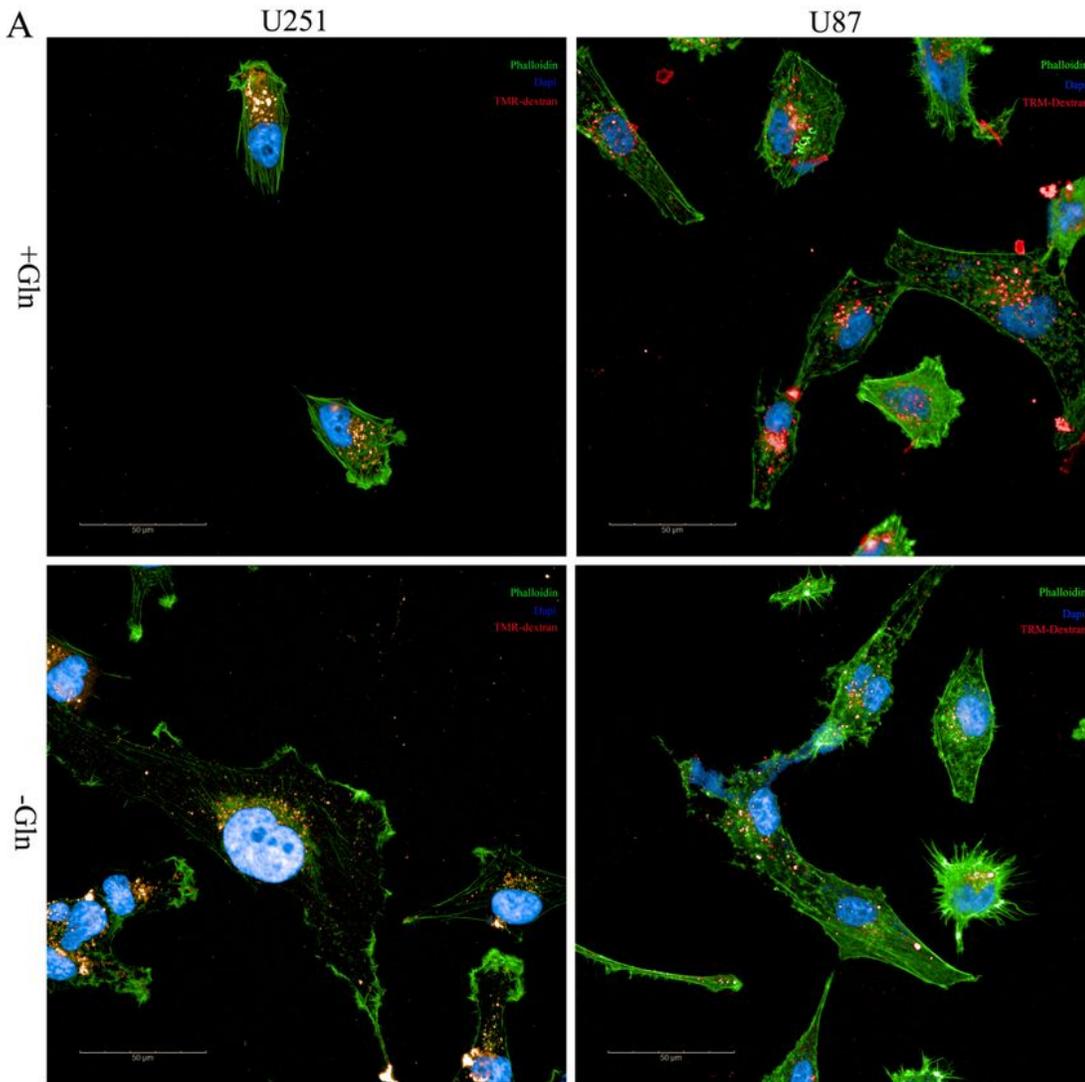


Figure 4

To determine whether stimulated macropinosocytosis is a feature of GBM exposing to nutrient stress, we compared TMR-dextran uptake of U251 and U87 cell lines in Gln deprivation medium and complete medium by the described protocol (Fig.4, A). Gln deprivation increased the macropinosome index in GBM cells (Fig.4, B and C).