FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68321
Scriptwriter Name: Poornima G
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Title: Exploring the Sequential Cellular Events of Phagocytosis Triggered by Godanti Bhasma in Mammalian Cells

Authors and Affiliations:
Gaurav Dutt, Vishakha Goswami, Abul Faiz, Alpana Joshi, Jayanand, Subrata K. Das

Shobhit Institute of Engineering & Technology

Corresponding Authors:
Subrata K. Das subrata.das@shobhituniversity.ac.in

Email Addresses for All Authors:
Gaurav Dutt gaurav.dutt@shobhituniversity.ac.in
Vishakha Goswami vishakha.goswami@shobhituniversity.ac.in
Abul Faiz abul.faiz@shobhituniversity.ac.in
Alpana Joshi alpana.joshi@shobhituniversity.ac.in
Jayanand jayanand@shobhituniversity.ac.in
Subrata K. Das subrata.das@shobhituniversity.ac.in

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar?**No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage?**Yes, done**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length
Number of Steps: 21
Number of Shots: 49 (8 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

NOTE: The file names and the god takes provided by the videographer are indicated at every shot. Please follow that.

1.1. **Subrata K. Das:**Phagocytosis, where phagocytes ingest and break down foreign particles, is crucial for immune defense. Improved, user-friendly methods are needed to efficiently monitor and evaluate this complex cellular process.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1* **Videographer's NOTE:** 1.1.1
C0005 Final Take

What are the current experimental challenges?

1.2. **Gaurav Dutt:**Capturing real-time degradation events, and avoiding artifacts from photobleaching remain key challenges in accurately studying phagocytosis and intracellular trafficking.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1* **Videographer's NOTE:** 1.2.1
C0012 Final Take

What advantage does your protocol offer compared to other techniques?

1.3. **Gaurav Dutt:**Unlike fluorescent or synthetic particles, our protocol uses Godanti Bhasma, a label-free particle, enabling direct visualization of phagocytosis stages without photobleaching, opsonization, or complex preparation.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.1* **Videographer's NOTE:** 1.3.1
C0013 Final Take

How will your findings advance research in your field?

1.4. **Subrata K. Das:**These findings provide a reproducible model to dissect phagocytic mechanisms, offering new tools to study vacuolar dynamics, phagosome dysfunction, and potential therapeutic interventions for immune and lysosomal disorders.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.2.1* **Videographer's NOTE:** 1.4.1
C0008 Final Take

What research questions will your laboratory focus on in the future?

1.5. **Subrata K. Das:**We aim to explore the molecular regulators of phagosome maturation, investigate phagocytic dysfunction in disease models, and screen potential drugs that modulate vacuolar dynamics and intracellular trafficking.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 7.3.1***Videographer's NOTE:** 1.5.1 C0062 Final Take

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of Godanti Bhasma (GB) Stock Solution for Cell Culture

Demonstrator: Vishakha Goswami

2.1. To begin, weigh exactly 100 milligrams of Godanti Bhasma or GB powder **[1]**. Suspend the powder in 10 milliliters of DMEM supplemented with 10 percent FBS and 1 percent penicillin-streptomycin **[2]**.

2.1.1. WIDE: Talent weighing 100 milligrams of GB powder on an analytical balance. **Videographer's NOTE:** 2.1.1 C0014 Final Take

2.1.2. Talent adding the GB powder to a container with 10 milliliters of supplemented DMEM. **Videographer's NOTE:** 2.1.2 C0015 Final Take

2.2. Vortex the suspension thoroughly to mix the contents **[1]**and let the mixture stand for 1 minute to allow the larger particles to settle at the bottom **[2]**. Using a pipette, transfer the upper 5 milliliters of the suspension into a sterile centrifuge tube **[3]**.

2.2.1. Talent vortexing the tube containing the GB suspension. **Videographer's NOTE:** 2.2.1 C0016 Final Take

2.2.2. Close-up of the tube left undisturbed for 1 minute to show settling particles. **Videographer's NOTE:** 2.2.2 C0017 Final Take

2.2.3. Talent pipetting the top 5 milliliters of the clear suspension into a new sterile centrifuge tube. **Videographer's NOTE:** 2.2.3 C0018 Final Take

2.3. Label the 5milliliters of transferred GB suspension as the stock solution for further cell culture work **[1]**. Vortex the suspension thoroughly again to ensure even dispersion of particles before adding it to the cells **[2]**.

2.3.1. Talent labeling the centrifuge tube as "GB stock solution". **Videographer's NOTE:** 2.3.1 C0019 Final Take

2.3.2. Talent vortexing the labeled tube again. **Videographer's NOTE:** 2.3.2 C0020 Final Take

3. Time-Lapse Microscopy of GB Treated Cells

3.1. Seed 3 x $10^4$3T3-L1 *(3-T-3-L-1)* cells into a 35-millimeter dish containing 2 milliliters of DMEM **[1]**. Incubate the dish at 37 degrees Celsius in an atmosphere of 5 percent carbon dioxide until the cells reach 70 percent confluency **[2]**.

3.1.1. Talent pipetting 3.0 x 10^4 3T3-L1 cells into a 35 millimeter culture dish with 2 milliliters of medium.**Videographer's NOTE:** 3.1.1 C0022 Final Take

3.1.2. Talent placing the culture dish in the carbon dioxide incubator.**Videographer's NOTE:** 3.1.2 C0023 Final Take

3.2. Then, mix 300 microliters of the GB stock suspension thoroughly into 2 milliliters of pre-warmed fresh culture medium **[1]**. Place the culture dish containing cells and GB suspension onto the stage incubator of the imaging microscope **[2]**.

3.2.1. Talent pipetting 300 microliters of GB stock into the medium and mixing thoroughly.**Videographer's NOTE:** 3.2.1 C0024 Final Take

3.2.2. Talent positioning the culture dish on the microscope stage Incubator.**Videographer's NOTE:** 3.2.2 C0025 Final Take

3.3. Select the **10x Objective** on the microscope **[1]**. Turn on the brightfield illumination using the **Bright** button **[2]**. Then launch the DIGIImageplus *(digi-image)* software and select the appropriate camera **[3]**. Click the **Capture** button and choose the **Time-lapse** option **[4]**. Set the interval time to 5 minutes **[5]** and start recording by clicking the **Finish** button **[6]**.

3.3.1. Talent adjusting the microscope to the 10x objective lens.**Videographer's NOTE:** 3.3.1 C0026 Final Take

3.3.2. Talent switching on the bright button manually on the microscope.
Videographer's NOTE: 3.3.2 C0027 Final Take

3.3.3. SCREEN: 68321_Screenshot_1.mp4 00:04-00:12.

3.3.4. SCREEN: 68321_Screenshot_2.mp4.

3.3.5. SCREEN: 68321_Screenshot_3.mp4.

3.3.6. SCREEN: 68321_Screenshot_4.mp4 00:05-00:11.

3.4. Capture brightfield images at 5-minute intervals continuously for 16 to 24 hours inside the microscope stage carbon dioxide incubator **[1]**.

3.4.1. Talent placing the culture dish inside the microscope stage incubator with

ongoing time-lapse capture visible on the screen.**Videographer's NOTE:** 3.4.1 C0029 Final Take

3.5. Use ImageJ software to compile all captured images into a time-lapse video **[1]**. This enables identification of the phagocytosis stages from particle internalization to their complete degradation **[2]**.

3.5.1. SCREEN: 68321_Screenshot_5.mp4. 00:55:01:06

3.5.2. SCREEN: 68321_Screenshot_6.mp4. 00:05-00:35 *Video editor Please speed up*

4. Neutral Red Staining of GB Treated Cells

4.1. After 24 hours of cell growth in 96-well plates, mix the GB particle stock suspension thoroughly **[1]**. Add 30 microliters of the suspension to each well **[2]**.

4.1.1. Talent vortexing the GB stock suspension before use. **Videographer's NOTE:** 4.1.1 C0031 Final Take

4.1.2. Talent pipetting 30 microliters of GB suspension into each well. **Videographer's NOTE:** 4.1.2 C0032 Final Take

4.2. Use wells without GB particles as negative controls **[1]**. Incubate the plates at 37 degrees Celsius in 5 percent carbon dioxide for 24 hours to induce vacuole formation **[2]**.

4.2.1 Talent marking the negative control well. **NOTE**: Shots are inverted and numbered as per new order

4.2.2 Talent placing the treated plates into a carbon dioxide incubator **Videographer's NOTE:** 4.2.2(earlier as 4.2.1) C0033 Final Take

4.3. To prepare Neutral Red solution, dissolve Neutral Red to make a 0.5 milligrams per milliliter solution in serum-free DMEM **[1]**. Filter the solution through a 0.2 micrometer filter **[2]**.

4.3.1. Talent dissolving Neutral Red powder in serum-free medium and mixing.**Videographer's NOTE:** 4.3.1 C0034 Final Take

4.3.2. Talent adding the dye solution into a 0.2 micrometer syringe filter. **Videographer's NOTE:** 4.3.2 C0035 Final Take

4.4. For imaging, remove the media from 96-well plates **[1]**. After adding 60 microliters of

the filtered dye to each well, incubate the plate at 37 degrees Celsius for 15 minutes **[2]**. Wash the wells three times with PBS to remove excess dye **[3]**, then proceed with microscopic imaging **[4]**.

4.4.1. Talent aspirating the media from each well. **Videographer's NOTE:** 4.4.1 C0036 Final Take

4.4.2. Talent placing the plate into the incubator. **Videographer's NOTE:** 4.4.2 C0037 Final Take

4.4.3. Talent adding the wells with PBS. **Videographer's NOTE:** 4.4.3 C0038 Final Take

4.4.4. Talent placing the stained samples under a microscope for imaging. **Videographer's NOTE:** 4.4.4 - 4.5.1C0040 Final Take

4.5. Examine the stained vacuoles under a microscope using the 20x objective **[1]** to assess the size, morphology, and number of vacuoles **[2]**.

4.5.1. Talent adjusting the microscope to the 20x objective and observing stained vacuoles. **Videographer's NOTE:** 4.4.4 - 4.5.1 C0040 Final Take

Added shot: SCREEN: Shot of NR staining on the screen while image capturing is in progress

Videographer's NOTE: (Added shot) C0041 Shot of the screen

5. Treatment with Bafilomycin A1 for Vacuole Formation and Acidification Inhibition

Demonstrator: Abul Faiz

5.1. To prepare Bafilomycin A1 stock solution, dissolve 100 micrograms of the compound in 50 microliters of dimethyl sulfoxide **[1]**, then dilute it with 950 microliters of DMEM **[2]**. For the working solution, dilute 1 microliter of the stock in 1.6 milliliters of DMEM to obtain a final concentration of 0.1 nanomolar **[3]**.

5.1.1. Talent dissolving 100 micrograms of Bafilomycin A1 in 50 microliters of dimethyl sulfoxide. **Videographer's NOTE:** 5.1.1 to 5.1.3 C0042 Final Take

5.1.2. Talent adding 950 microliters of DMEM to the dissolved solution and shaking the tube.

5.1.3. Talent pipetting 1 microliter of stock into 1.6 milliliters of medium to prepare the working solution.

5.2. Mix the working solution thoroughly **[1]** and add 100 microliters to the wells containing cells in the 96-well plates **[2]**. Then, incubate the cells in each well with 30 microliters

of GB particle suspension to study inhibition of vacuole and phagosome formation **[3]**.

5.2.1. Talent vortexing or pipetting to mix the Bafilomycin A1 working solution. **Videographer's NOTE:** 5.2.1 C0043 Final Take

5.2.2. Talent pipetting 100 microliters of the Bafilomycin A1 solution and 30 microliters of GB into cell-containing wells. **NOTE:** May be C0044 Final Take

5.2.3. Talent placing the plate aside for incubation. **NOTE:** may be C0045 Final Take

5.3. To inhibit phagosome acidification, add 100 microliters of the Bafilomycin A1 working solution to wells with pre-formed vacuoles previously treated with GB **[1]**. Incubate all wells at 37 degrees Celsius in 5 percent carbon dioxide for 24 hours **[2]**.

5.3.1. Talent pipetting 100 microliters of the Bafilomycin A1 working solution into wells with pre-formed vacuoles. **Videographer's NOTE:** 5.3.1 C0046 Final Take

5.3.2. Talent placing the treated plate back into the incubator. **Videographer's NOTE:** 5.3.2 C0047 Final Take

5.4. Add 60 microliters of Neutral Red dye to each well **[1]**. Incubate for 15 minutes at 37 degrees Celsius **[2]** and wash the wells three times with PBS to remove excess dye **[3]**, then analyze vacuole formation and acidification under the microscope **[4** and **5]**.

5.4.1. Talent pipetting 60 microliters of dye into the wells. **Videographer's NOTE:** 5.4.1 C0048 Final Take

5.4.2. Talent placing the plate into the incubator. **Videographer's NOTE:** 5.4.2 C0047 Same as 5.3.2

Added shot: Talent adding the wells with PBS. **Videographer's NOTE:** 5.4.3(Added shot) C0049

5.4.3 Talent placing the sample under the microscope. **Videographer's NOTE:** 5.4.4(Earlier as 5.4.3) C0050

Added shot : SCREEN: Show Vacuole inhibition and Vacuole Acidification inhibition. **Videographer's NOTE:** C0051 b) Vacule inhibition result C0052 c) Acidification inhibition . *Please show 5.4.3 and this added shots using split screen*

6. Acridine Orange (AO) Staining to Assess the Cells Post-Treatment

Demonstrator: Gaurav Dutt

6.1. Treat the cultured cells with 50 microliters of GB suspension **[1]**and incubate them overnight at 37 degrees Celsius in 5 percent carbon dioxide under standard experimental conditions **[2]**.

6.1.1. Talent pipetting 50 microliters of GB suspension into the culture. **Videographer's NOTE:** 6.1.1 C0053 Final Take

6.1.2. Talent placing the sample in the carbon dioxide incubator. **Videographer's NOTE:** 6.1.2 C0054 Final Take

6.2. Following treatment, add acridine orange solution at 1 milligram per milliliter concentration to the cells **[1]** and incubate for 15 minutes at 37 degrees Celsius to stain vacuoles and acidic vesicular organelles **[2]**.

6.2.1. Talent adding acridine orange solution to each cavity. **Videographer's NOTE:** 6.2.1 C0055 Final Take

6.2.2. Talent placing the slides in the incubator. **Videographer's NOTE:** 6.2.2 C0053 Same as 6.1.1

6.3. Next, wash the cells three times with sterile PBS to remove any unbound dye and reduce background fluorescence **[1]**.

6.3.1. Talent adding the stained slides with PBS using a pipette. **Videographer's NOTE:** 6.3.1 C0056 Final Take

6.4. Finally, analyze the stained slides immediately using a fluorescence microscope fitted with a 20x objective and a blue excitation filter range of 450 to 492 nanometers**[1]**. Assess cellular morphology and acridine orange uptake to understand vacuolar dynamics **[2]**.

6.4.1. Talent placing the slide under the fluorescence microscope and adjusting to the 20x objective. **Videographer's NOTE:** 6.4.1 C0057 Final Take

6.4.2. SCREEN: 68321_Screenshot_7.mp4. 00:20-00:31

Results

7. Results

7.1. After 30 minutes of GB treatment, particles were observed attached to the surface of 3T3-L1 cells **[1]**.A cup-like membrane extension appeared around the particle, indicating the initiation of particle engulfment**[2]**.

7.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight 1ST image on row 2, and focus on the black spot at the cell boundary*.

7.1.2. LAB MEDIA: Figure 1. *Video editor:Highlight 1ST image on row 3, and focus on the black spot that forms cup like structure*.

7.2. At 12 hours post-treatment, large membrane-bound vacuoles containing GB particles were observed inside the cells **[1]**.

7.2.1. LAB MEDIA: Figure 1D. *Video editor: Highlight the 4TH row images sequentially*.

7.3. Time-lapse imaging demonstrated the stepwise internalization and vacuole formation in a single live cell over 4.75 hours **[1]**.

7.3.1. LAB MEDIA: Figure 2. *Video editor: Sequentially highlight the images and focus on the cells at the center (very light round cell which is eating up some black particles)*.

7.4. GB particles within vacuoles progressively degraded over time, and the cell regained normal morphology, as indicated by vacuolar turnover **[1]**.

7.4.1. LAB MEDIA: Figure 3. *Video editor: Sequentially highlight the images*.

7.5. Flow cytometry analysis showed that internalized Godanti Bhasma particles were completely degraded within 24 hours, with no particles remaining in the culture medium **[1]**.

7.5.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the 24 h Images*.

7.6. Neutral Red staining revealed significantly higher vacuolar acidification in GB-treated cells of all three cell lines compared to**[1]** controls, which displayed only weak lysosomal staining **[2]**.

7.6.1. LAB MEDIA: Figure 5A-C. *Video editor: Highlight the large red-stained vacuoles in each GB-treated cell image*.

7.6.2. LAB MEDIA: Figure 5D-F.

7.7. Acridine Orange staining showed orange-red fluorescence in vacuoles of GB-treated 3T3-L1 cells, indicating acidification **[1]**, while untreated cells showed only green fluorescence **[2]**.

7.7.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the vacuoles showing orange-red coloration in treated cells*.

7.7.2. LAB MEDIA: Figure 6B.

7.8. Co-treatment with Bafilomycin inhibited vacuole formation in both 3T3-L1 **[1]** and HeLa cells, as evidenced by the absence of vacuoles**[2]** compared to their respective controls **[3]**.Aftertreatment with Bafilomycin, Neutral Red uptake was effectively blocked, disrupting vacuole acidification and maturation in cells**[4]**.

7.8.1. LAB MEDIA: Figure 7A.

7.8.2. LAB MEDIA: Figure 7C.

7.8.3. LAB MEDIA: Figure 7B and D. *Video editor: Highlight the visible RED vacuoles*.

7.8.4. LAB MEDIA: Figure 8*Video editor: Highlight A and C*.

- **Godanti Bhasma**

Pronunciation link (HowToPronounce):
https://www.howtopronounce.com/patanjali-divya-godanti-bhasma
howtopronounce.com+15howtopronounce.com+15howtopronounce.com+15
IPA: /goʊˈdʌn.ti ˈbɑːʃ.mɑː/
Phonetic: go-DUN-tee BAHSH-mah

- **DMEM** (Dulbecco's Modified Eagle Medium)

No confirmed pronunciation link found
IPA: /diː-ɛm-ɛm/
Phonetic: dee-em-em

- **FBS** (Fetal Bovine Serum)

No confirmed link found

IPA: /ɛf-bi-ɛs/
Phonetic: ef-bee-ess

- **Penicillin–Streptomycin**

Pronunciation link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/penicillin
https://www.merriam-webster.com/dictionary/streptomycin
IPA: /ˌpɛnɪˈsɪlɪn strɛpˈtoʊmaɪsɪn/
Phonetic: pen-ih-SILL-in strep-TOE-my-sin

- **Centrifuge tube**

Pronunciation link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛn.trə.fjuːdʒ tuːb/
Phonetic: SEN-truh-fyooj toob

- **Confluency**

Link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/confluency
IPA: /ˈkɒn.flu.ən.si/
Phonetic: KON-floo-en-see

- **Incubator**

Link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/incubator
IPA: /ˈɪŋ.kjə.beɪ.tər/
Phonetic: ING-kyu-bay-tur

- **Time-lapse**

Link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/time-lapse
IPA: /ˈtaɪmˌlæps/
Phonetic: TYME-laps

- **Brightfield** (microscopy mode)

No confirmed link found
IPA: /ˈbraɪtˌfiːld/
Phonetic: BRYTE-feeld

- **Acridine orange**

Link (HowToPronounce doesn't exist; using Merriam-Webster for “acridine”):
https://www.merriam-webster.com/dictionary/acridine
IPA: /ˈæk.rɪˌdiːn ˈɔːr.ɪndʒ/
Phonetic: ACK-ri-deen OR-inj

- **Vacuolation**

Link (No confirmed source found)
IPA: /ˌvæk.ju.əˈleɪ.ʃən/
Phonetic: vak-yoo-ah-LAY-shun

- **Phagocytosis**

Link (Merriam-Webster):
https://www.merriam-webster.com/dictionary/phagocytosis
IPA: /ˌfæg.oʊ.saɪˈtoʊ.sɪs/
Phonetic: fag-oh-sigh-TOE-sis

- **Bafilomycin A₁**

No pronunciation link found
IPA: /bəˌfɪl.əˈmaɪ.sɪn eɪ wʌn/
Phonetic: ba-FIL-oh-MY-sin A-one

- **Neutral Red** (dye name)

Link (Merriam-Webster for “neutral” and “red”):
https://www.merriam-webster.com/dictionary/neutral
https://www.merriam-webster.com/dictionary/red
IPA: /ˈnjuː.trəl rɛd/
Phonetic: NEW-truhl red

- **Analytical balance**

Link for “analytical”:
https://www.merriam-webster.com/dictionary/analytical
IPA: /ˌæn.əˈlɪt.ɪ.kəl ˈbæl.əns/
Phonetic: an-uh-LIT-i-kul BAL-uns