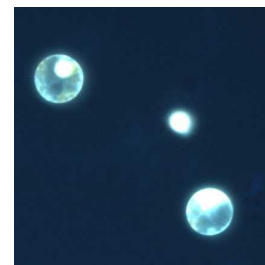


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Time-course live imaging of maize and sorghum protoplasts

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Rachel Baschieri¹, Thai Dao¹, Samuel Leiboff¹

¹Oregon State University

Time-course live imagin...



Rachel Baschieri

Oregon State University

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Protocol status: Working

We use this protocol and it's working

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Abstract

A protoplast is a living plant, fungal, or bacterial cell with the cell wall removed. Protoplasts offer a simplified system for studies of gene expression compared to more complex whole-plant systems and are promising for the interrogation of cellular physiology, metabolism and responses to stimuli. Performing live imaging of protoplast experiments in a time-course experiment provides more information about how these cellular processes progress over time. Protoplast time-course live imaging studies have previously explored intracellular auxin localization [1], auxin's effect on gene expression [2], the cellular localization of a mobile transcription factor, and its dynamic response to chemical endomembrane system inhibitors [3], cell wall regeneration [4], and cell division and microcallus formation [5].

Though time-course live imaging of protoplasts is a useful tool, it is also a skill that can be difficult to acquire. Practice and troubleshooting of various protoplast isolation, transfection, and immobilization protocols can be time-consuming and may deter many from performing experiments with protoplasts. This protocol combines protoplast isolation, transfection, and immobilization methods to form a cohesive protoplast time-course live imaging protocol with notes and modifications from our troubleshooting.

This protocol was developed for time-course live imaging of maize and sorghum protoplasts. The isolation and transfection steps are based on methods published in Meng et al. 2020 with a few modifications. First, 50mM MES was added to the PEG-calcium transfection solution to buffer the transfection solution at pH 5.7 and increase protoplast transfection efficiency. Second, 1% bovine serum albumin(BSA) was added to the incubation solution based on Ohshima et al. 1989 to increase protoplast viability after overnight incubation. Third, 50ug/mL ampicillin is added to the incubation solution to decrease bacterial growth overnight [8]. The alginate immobilization steps were adapted from Middleton et al. 2018 and Dovzhenko et al. 1998.

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Materials

Enzyme solution - pH 5.7

- 10mM KCL
- 8mM MES
- 1mM CaCl₂
- 0.5M mannitol
- 0.6% cellulase
- 0.1% pectolyase (macroenzyme)
- 0.1% BSA
- 0.1% polyvinylpyrrolidone K30

	A	B
	Stock	Amount for 10mL
	0.1M KCL	1 mL
	0.1M MES	800 uL
	1M CaCl ₂	10 uL
	1M mannitol	5 mL
	Sterile ddH ₂ O	3.19 mL
	Cellulase RS	0.06 g
	Macroenzyme R-10	0.01 g
	BSA	0.01 g
	Polyvinylpyrrolidone K30	0.01 g

Filter sterilize Enzyme solution

W5 solution - pH 5.7

- 154 mM NaCl
- 125 mM CaCl₂
- 5 mM KCl
- 2 mM MES

	A	B	C	D
	Stock	15mL	20 mL	30 mL

	A	B	C	D
	NaCl	0.1349964 g	0.1799952 g	0.2699928 g
	1M CaCl ₂	1.875 mL	2.5 mL	3.75 mL
	0.1M KCl	750 uL	1 mL	1.5 mL
	0.1M MES	300 uL	400 uL	600 uL
	Sterile ddH ₂ O	12.075 mL	16.1 mL	24.15 mL

Filter sterilize W5 solution

Suspension solution - pH 5.7

- 0.4M mannitol
- 20 mM CaCl₂
- 5 mM MES

	A	B	C	D
	Stock	1 mL	5 mL	15mL
	1M mannitol	400 uL	2,000 uL	6 mL
	1M CaCl ₂	20 uL	100 uL	300 uL
	0.1M MES	50 uL	250 uL	750 uL
	Sterile ddH ₂ O	530 uL	2,650 uL	7,950 uL

Filter sterilize Suspension solution

PEG solution - pH 5.7

- 40% PEG 4000
- 0.1M CaCl₂
- 0.4M mannitol, pH 5.7
- 50mM MES

	A	B	C
	Stock	1 mL	3 mL
	0.1M MES	500 uL	1,500 uL
	1M CaCl ₂	100 uL	300 uL



	A	B	C
	1M mannitol	400 uL	1200 uL
	PEG4000	0.4g	1.2g

Filter sterilize PEG solution

Incubation solution. - pH 5.7

- 0.5M mannitol
- 4 mM KCl
- 4 mM MES
- 1% BSA
- 50ug/ml Ampicillin

	A	B	C	D	E
	Stock	3mL	6mL	10 mL	15 mL
	1M mannitol	1.5 mL	3 mL	5 mL	7.5 mL
	0.1M KCL	120 uL	240 uL	400 uL	600 uL
	0.1M MES	120 uL	240 uL	400 uL	600 uL
	Sterile ddH ₂ O	1.26 mL	2.52 mL	4.2 mL	6.3 mL
	BSA	0.03g	0.06 g	0.1 g	0.15g
	Ampicillin 100mg/ml	1.5ul	3ul	5ul	7.5ul

Filter sterilize Incubation solution

MMM pH-5.7

	A	B
	Reagent	Amount to make 100mL
	MgCl ₂ Hexahydrate	102mg
	MgSO ₄ Heptahydrate	125mg

	A	B
	Mannitol	8.5g
	MES	195.2mg

Autoclave MMM solution

Alg-A pH -5.7

	A	B
	Reagent	Amount to make 100mL
	MgCl ₂ Hexahydrate	102mg
	MgSO ₄ Heptahydrate	125mg
	Mannitol	8.5g
	MES	195.2mg
	Alginic acid	1.2g

Filter sterilize Alg-A solution

2M NH₄-Succinate

	A	B
	Reagent	Amount to make 200mL
	NH ₄ Cl	21.2g
	KOH	44.8g
	Succinic acid	47.2g

Filter sterilize 2M NH₄-Succinate

FPCN pH-5.7



	A	B
	Reagent	Amount to make 50mL
	KNO ₃	0.0506g
	CaCl dihydrate	0.032g
	MgSO ₄ Heptahydrate	0.0185g
	KH ₂ PO ₄	0.0085g
	MS	0.220255g
	NH ₄ -succinate	500uL
	Inositol	0.01g
	Sucrose	1g
	Glucose	4g
	MES	0.0976g
	10mM Biotin	0.2uL
	100mM Thiamine-HCl	1.5uL
	100mM Nicotinic Acid	8uL

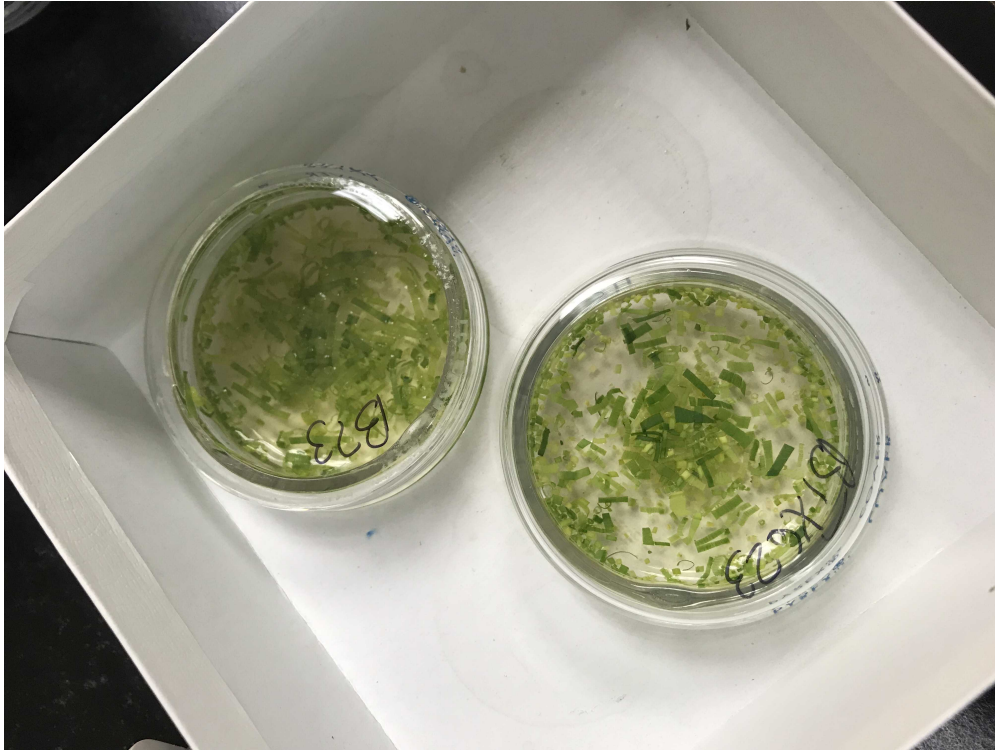
Filter sterilize FPCN solution

Day 1 - Protoplast Isolation and Transfection

- 1 Prepare enzyme solution.
- 2 Harvest 10-14 day old seedlings and submerge in 5% bleach 1% Tween solution for 1min., remove and rinse seedlings thoroughly in autoclaved tap water 6x. Pat seedlings dry with autoclaved Kimwipes.
- 3 Fill a glass petri dish with the filter sterilized enzyme solution, and set aside.
- 4 Cut green leaf tissue from seedlings into ~1mm strips with a fresh razor blade and immediately transfer to glass petri dish pre-filled with filter sterilized enzyme solution.

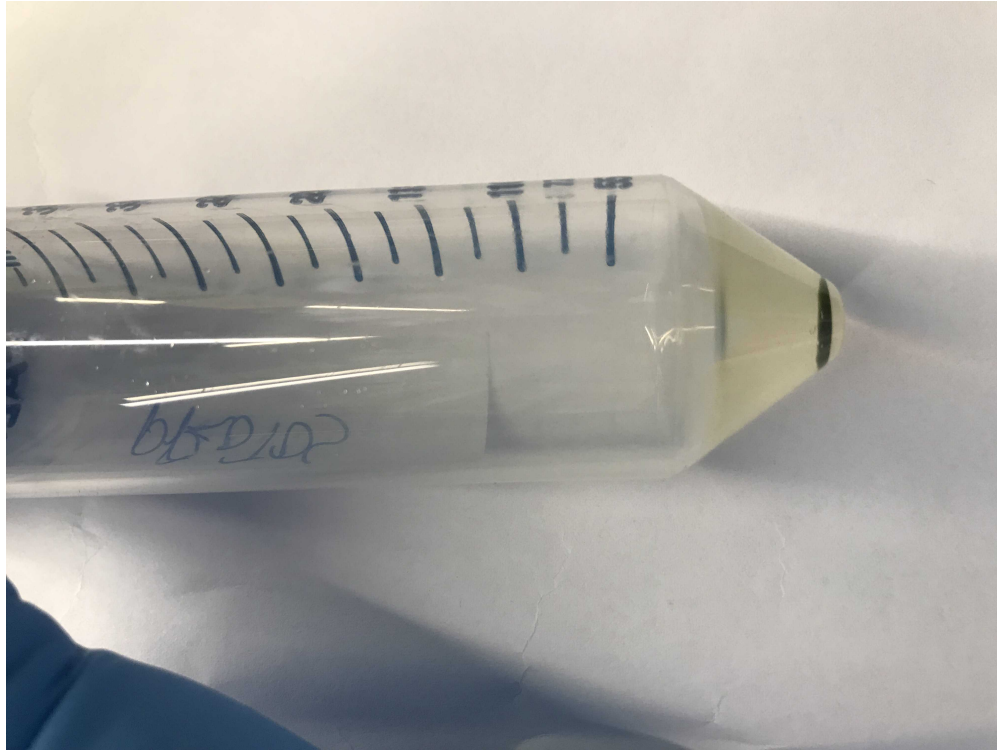


Thinly sliced sorghum leaf tissue.



Maize B73 and sorghum BTX623 leaf tissue in enzyme solution.

- 5 Incubate 4h in dark at RT w/ 40 rpm shaking.
- 6 Prepare W5 solution, suspension solution, PEG solution, and incubation solution.
- 7 Add 10mL W5 solution (or volume of W5 equal to the amount of enzyme solution). Gently swirl to mix, and shake additional 1h at 80 rpm.
- 8 Filter the W5 and enzyme solution mixture through 100 or 70um nylon mesh into 50 mL tube.
- 9 Centrifuge @ 1000-1200 rpm 5 min to collect protoplasts to the bottom of the 50mL tube.



- 10 Use a pipette to remove the supernatant and resuspend the protoplast pellet in 600 µL of suspension solution if working on sorghum, 1ml of suspension solution if working on maize.
- 11 Use 9µl of resuspended protoplasts to count with a hemocytometer to determine the concentration of resuspended protoplasts.



B73_7_for_protocol.jpg



BTX623_4_for_protocol.jpg

STEP CASE

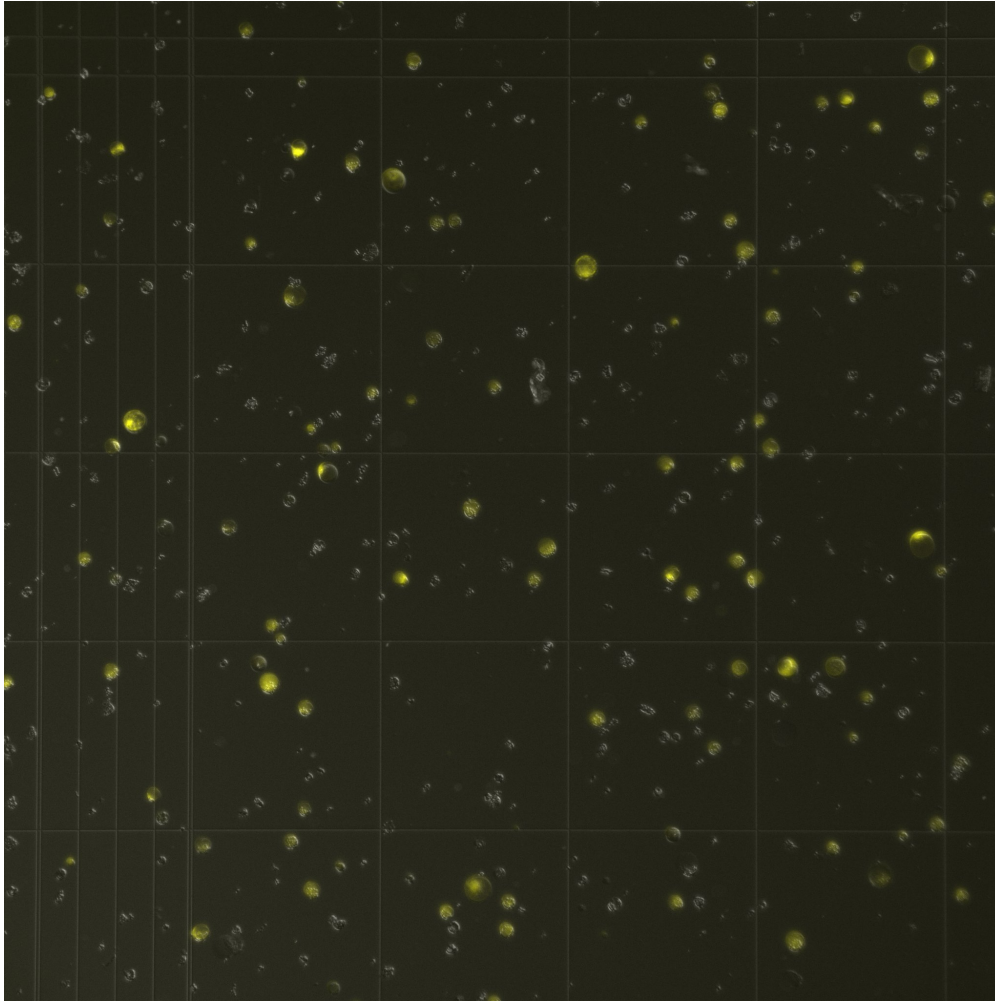
Fluorescein Diacetate (FDA) viability stain

18 steps

FDA stain to check viability of protoplasts:

- 81.2µL 1M mannitol
- 43.6µL diH₂O
- 2.5µL FDA

Mix 10µL protoplast suspension with 5µL FDA stain, incubate RT 2min., load on hemocytometer



Sorghum BTX623 protoplasts FDA stained

- 12 Add more suspension solution to reduce protoplast concentration to $\sim 2-4 \times 10^5$ cells/mL for transfection.
- 13 In a 1.5mL tube add 20-40ug plasmid DNA and 200uL of protoplast suspension, let sit RT 5 min.
- 14 Add 220uL PEG solution and mix immediately by gentle tube inversion, incubate 15 min. @ 28C.
- 15 Quickly add 800 uL W5 solution to dilute the PEG, gently invert the tube 2x.



- 16 Collect the protoplasts by centrifuge 3min. @ 1000rpm, remove PEG and W5 supernatant.
- 17 Suspend collected protoplasts in 1mL incubation buffer and incubate in the dark @ 28C overnight with gentle ~20rpm shaking. 1.5mL tubes should be laid on their side for overnight incubation.
- 18 Ensure MMM, Alg-A, and FPCN solutions are prepared for Day 2. The MMM and Alg-A solutions may be stored at 4C for 6 months or longer, FPCN may be stored at 20C for 6 months or longer.

Day 2- Protoplast Immobilization in well-plate

- 19 In a sterile hood, coat the bottom surface of the slide-well the protoplasts will be sitting in with 50ug/mL Poly-L-lysine, allow to sit for 30min-1hour, remove the poly-L-lysine, and allow the well-plate to air-dry.
- 20 Centrifuge transformed protoplasts after overnight incubation @ 1000rpm for 3minutes.
- 21 Remove the incubation buffer supernatant, resuspend the protoplast pellet in 100uL MMM and 100uL Alg-A solution. Mix by gently flicking the 1.5mL tube.
- 22 Pipette a large droplet of the protoplast solution onto Poly-L-lysine coated slide well (we use 8 well chamber, 50uL protoplast droplet in each well)
- 23 Allow the alginate droplet to sit for 10-15 minutes at RT for the protoplasts to settle to the surface of the glass slide.
- 24 Gently pipette 0.5-1uL droplets of W5 solution to the surface of the protoplast-alginate droplet so that the entire surface of the droplet gets coated in W5, but the protoplasts settled against the glass remain undisturbed as possible.



First solidify the outer edges of the protoplast-alginate droplet with microdroplets of W5, then work inward.

- 25 Allow the W5 coated protoplast-alginate droplet to sit 15 minutes RT.
- 26 Pipette the equivalent volume of W5 as the volume of the protoplast-alginate droplet into the slide well containing the droplet (50uL W5: 50uL protoplast-alginate) and allow it to incubate for 45 minutes. Note: while pipetting, depress the W5 into the well very slowly and gently.
- 27 Remove W5 and incubate the droplet in FPCN (350uL FPCN if protoplast droplet is 50uL) for 15 minutes. This is wash 1.
- 28 Remove FPCN and replace with another addition of fresh FPCN, incubate 15 minutes. This is wash 2.
- 29 Remove FPCN and incubate with the final FPCN or treatment solution. The alginate-embedded protoplasts are ready to be imaged.



Maize and sorghum solid protoplast-alginate droplets in FPCN solution on 8 well slide.



24hour time-course of maize protoplasts transformed with a fluorescent construct.

