Cah. Biol. Mar. (2013) 54 : 625-631

Assessing fluorochrome-staining efficacy in the green sea urchin, *Strongylocentrotus droebachiensis* (Müller, 1776)

Nathan HAAG[1], Michael P. RUSSELL[1], Jose Carlos HERNANDEZ[2] and Norman DOLLAHON[1]
(1) *Biology Department, Villanova University, Villanova, PA 19085, USA*
Fax: 001 610 519 7863, E-mail: Michael.Russell@villanova.edu
(2) *Dpto. Biología Animal (Ciencias Marinas), Biodiversidad, Ecología Marina y Conservación, Universidad de La Laguna, Islas Canarias, Spain*

Abstract: Fluorochrome tagging is useful in growth, reproductive, and behavior studies. One commonly used fluorochrome, calcein, is typically administered by immersing individuals in a bath containing dissolved calcein. However, there are no optimal dosing studies for echinoderms. We used a 3 x 4-factorial (ANOVA) experiment to test whether lower concentrations of calcein (20 $mg \cdot l^{-1}$ = 100%, 10 $mg \cdot l^{-1}$ = 50%, 2 $mg \cdot l^{-1}$ = 10%) and shorter soaking times (24 h, 12 h, 4 h & 2 h) produced detectable fluorescence in the green sea urchin *Strongylocentrotus droebachiensis* (Müller, 1776). Juvenile sea urchins (n = 72) from a single hatchery spawning cohort were standardized by size (mean and variance) into 12 groups. These groups were randomly assigned to the 12 factorial treatments and individual replicates randomly distributed in a circulating sea table. During calcein treatment, individual urchin replicates were isolated in 500 ml beakers, maintained at a constant temperature, and returned to the sea table after 24 hours. Replicates were then kept in controlled conditions for ten days after the treatment. Urchins were then euthanized, processed with sodium hypochlorite, rinsed, and dried. We examined the demipyramids of Aristotle's lantern with UV illumination and quantified the intensity of fluorescent marks with ImageJ software. Demipyramids from replicates soaked in 50% for 12 hours were not significantly different from those in 100% at 24 hours. This lower exposure treatment combination may ameliorate any negative effects of calcein exposure.

Résumé : *Evaluation de l'efficacité du marquage par fluorochrome chez l'oursin vert* Strongylocentrotus droebachiensis *(Müller, 1776).* Le marquage par fluorochrome est utile dans les études de croissance, de reproduction et de comportement. Un fluorochrome couramment utilisé, la calcéine, est administré par bain de trempage. Cependant, aucune étude des dosages optimaux n'existe pour les échinodermes. Nous avons utilisé une analyse de variance (ANOVA) pour tester si des concentrations plus faibles de calcéine (20 $mg \cdot l^{-1}$ = 100%, 10 $mg \cdot l^{-1}$ = 50%, 2 $mg \cdot l^{-1}$ = 10%) et diminution des temps de trempage (24 h, 12 h, 4 h & 2 h) produisent une fluorescence détectable dans l'oursin vert *Strongylocentrotus droebachiensis* (Müller, 1776). Septante deux oursins juvéniles, issus d'une cohorte unique de ponte en écloserie, ont été répartis au hasard parmi les 12 traitements qui ont été maintenus dans un système circulaire d'aquariums. Pendant le traitement à la calcéine, chaque groupe d'oursin a été isolés dans des béchers de 500 ml, maintenu à une température constante, et est retourné dans le système circulaire d'aquariums au bout de 24 heures. Les oursins ont été remis en conditions contrôles pendant dix jours. Ils sont ensuite euthanasiés et les tests sont traités avec de l'hypochlorite de sodium, rincés et séchés. Les demi-pyramides de la lanterne d'Aristote ont été examinées sous une illumination UV en vue de quantifier l'intensité des marques fluorescentes avec le logiciel Image J. Les marquages des demi-pyramides des oursins trempées dans une solution de calcéine à 50% pendant 12 heures ne sont pas significativement différents de ceux des spécimens immergés dans une solution 100% pendant 24 heures. Cette incubation de plus courte durée et moins concentrée permettrait de minimiser les effets négatifs éventuels du traitement à la calcéine.

Keywords: Sea urchin ● Fluorochrome ● Calcein ● Life history ● Growth studies ● Aristotle's Lantern

Introduction

First developed by Kobayashi & Taki (1969), fluorochrome marking has enhanced our understanding of ossicle growth dynamics (Kobayashi & Taki, 1969; Pearse & Pearse, 1975; Gage, 1992; Ebert, 1999; Russell & Meredith, 2000) resource allocation (Russell, 1987), growth modeling (Lamare & Mladenov, 2000; Rogers-Bennett, 2003), movement (Dumont et al., 2004) and life table estimates (Ebert & Russell, 1992; Ebert, 1999). Accurately estimating these growth patterns is necessary to understanding echinoid life histories and population dynamics. These data are especially important for resource management and sustaining populations that are commercially exploited. The green sea urchin, *Strongylocentrotus droebachiensis* (Müller, 1776), is of particular interest because of its central ecological role in nearshore communities and it is the target of an active fishery in both the Atlantic and Pacific oceans.

Studies using two common carbonate fluorochromes, tetracycline and calcein, have revealed that: (1) some species can be slow growing and long-lived (Pearse & Pearse, 1975; Ebert, 1999; Lamare & Mladenov, 2000; Rogers-Bennett, 2003) and (2) natural growth lines in ossicles are not added annually and are invalid estimators of age, "chronometers" *sensu* Pearse & Pearse (Pearse & Pearse, 1975; Ebert, 1988; Gage, 1992; Russell & Meredith, 2000). Further, both compounds have been shown to have minimal effect on long term sea urchin growth (Ebert, 1988; Russell & Urbaniak, 2004; Ellers & Johnson, 2009; Sonnenholzner et al., 2010). However, because calcein is more brightly fluorescent, more easily absorbed, and less toxic, it is more commonly used than tetracycline (Rowley & Mackinnon, 1995).

Calcein is administered either by soaking (absorption into the coelom via osmosis) or by injection through the peristomal membrane, and binds irreversibly to calcium ions in developing skeletal material (Kobayashi & Taki, 1969; Pearse & Pearse, 1975; Rowley, 1990; Gage, 1992; Rowley & Mackinnon, 1995; Ebert, 1999; Lamare & Mladenov, 2000; Russell & Meredith, 2000; Rogers-Bennett, 2003). To assess growth, tagged sea urchins are normally left in the field and collected the following year. Samples are then processed by removing the viscera and the skeletal elements cleansed of soft tissue by soaking in sodium hypochlorite. The demipyramids from Aristotle's Lantern are normally used to quantify growth. Illumination with UV light clearly reveals the fluorescent stain and indicates the size of the demipyramid at the time of tagging.

In addition to sea urchins, calcein has been used in several tagging studies of other marine invertebrates including mussels (*Perna perna* (Linnaeus, 1758), Kaehler, 1999), sea cucumbers (*Holothuria scabra* (Jaeger 1833), Purcell et al., 2006; Purcell & Blockmans, 2009), abalone (*Haliotis rubra* (Leach, 1814), Day et al., 1995), and limpets *Siphonaria australis* (Quoy & Gaimard, 1833), Fitzpatrick et al., 2010, see Table 1). Fluorochromes have also been used to examine seasonal growth in otoliths in both marine and freshwater fish: Sciaenidae sp. (Wilson & Beckman, 1987), Salmonids sp. (Niva et al., 2005; Nagiec et al., 2008), Percidae sp. (Niva et al., 2005), and Clariidae sp. (Weyl & Booth, 2008). In these taxa, fluorochrome studies have progressed our understanding of growth and life histories (Wilson & Beckman, 1987; Day et al., 1995; Kaehler, 1999; Weyl & Booth, 2008; Fitzpatrick et al., 2010) as well as fisheries and hatcheries management (Niva et al., 2005; Purcell et al., 2006; Nagiec et al., 2008; Purcell & Blockmans, 2009). However, while other taxa have been tested for optimal calcein treatments (Purcell et al., 2006; Purcell & Blockmans, 2009), echinoids have not. The goal of this experiment was to determine the efficacy of various combinations of calcein concentration and soaking times to optimize processing, save resources, and ultimately, better understand sea urchin growth.

Methods

Adult sea urchins were collected from the mouth of the Piscataqua River in the Gulf of Maine, USA, and spawned on April 21, 2007. The larvae were raised and settled in a hatchery at the University of New Hampshire. Juveniles were shipped to Villanova University 10 months later on February 19, 2008 and immediately placed in a 1,000 liter re-circulating seawater system housed in an environmental chamber maintained at 10°C (salinity between 31-32). We recorded weight as well as test height and diameter. We selected 72 urchins with a test diameter range of 11.35-18.26 mm (mean ± SD = 14.95 ± 2.10) and randomly distributed them in a grid of individual cages suspended in a sea table. Each 200 ml cylindrical acrylic cage had an open top (the rim above the water line) and a 5 mm mesh-bottom that allowed vigorous aeration from the airstones positioned below on the bottom of the sea table. Each urchin was fed *ad-libitum* strips of the kelp *Macrocystis pyrifera* (Linnaeus) changed daily.

The experiment was a 3 x 4-factorial ANOVA with calcein concentration as one factor with 3 levels (100%, 50%, 10% of a stock solution at 20 mg l^{-1}) and soaking time as the other factor with 4 levels (24 h, 12 h, 4 h & 2 h). We assigned the sea urchins to 12 groups of 6 replicates so the average sizes and variances were approximately equivalent. These 12 groups were randomly assigned a treatment combination of concentration x soaking time. Another group of six urchins served as a control and was not exposed to calcein. This control group quantified the "natural levels" of fluorescence using the gray-scale method described below.

We allowed the urchins to acclimate to the system for 10

Table 1. A selection of studies using fluorochrome solutions to assess growth in marine animals. Listed are the taxa tagged, fluorochrome tag (C = calcein, T = tetracycline), method of tag (I = injection, S = soaking), and tag solution.

Taxa	Study	Tag	Method	Solution
Brachiopods, Bryozoans, Gastropods, Polychaetes, Bivalves, Cephalopods, Ophiuroids, Echinoids, Asteroids, Holothuroids, Crustaceans	Rowley 1995	C	IS	I: 10ml calcein/kg body wt; S:500 mg calcein/1Lsea -
Sciaenops ocellatus, Micropogonias undulatus, Leiostomus xanthurus	Wilson 1987	CT	S	S: 6.25g calcein/L tap S: 250mg tetracycline/L tap
Holothuria scabra - sea cucumber	Purcell 2006	CT	IS	I: 0.5 to 0.8 mL of 200 mg/L sln; S:200 mg/L for 21 h
Perna perna - brown mussel	Kaehler 1999	C	IS	I: 0,12,20,40,80,160,320,640 mg/L S: 150 and 500 mg, 4 h
Echinus esculentus	Gage 1992	T	I	I: 1 mg/ 0.1ml sea, 0.1 ml per 10g body weight
Evichinus chloroticus	Lamare 2000	C	IS	I: 500 ppm S: 500 ppm @ 5 and 12h.
Strongylocentrotus droebachiensis	Russell 2000	C	IS	I: 0.1-1 ml of 1 g/100ml sea sln; S: 0.625g calcein/100mL sea, 24 hrs
S. franciscanus	Ebert 1992	T	I	I: 0.2 ml of 1 g/100 ml sea sln
S. franciscanus	Rogers-Bennett 2003	T	IS	I: 1 g/100ml sea S: 125mg/L sea for 24 hrs
S. intermedius	Kobayashi 1969	T	I	I: 100 mg tetracycline per kg body weight
S. purpuratus	Rowley 1990	T	IS	I: 0.01 to 0.05 ml of 2g/L sea sln S: 0.05g/L sea
S. purpuratus	Russell 1987	T	I	I: 0.2 ml of 1 g/100 ml sea sln
S. purpuratus and *Echinometra mathaei*	Ebert 1988	T	I	I: 2, 4 and 8 mg in 0.1mL sear

days to insure active growth and allow for successful tagging. To evaluate growth and tagging potential, we selected two additional urchins for a trial. On February 29, 2008, we immersed these two trial individuals in 300 ml of the 100% calcein stock solution (20 $mg \cdot l^{-1}$) separate from the system for 24 hours. After the tagging, we vigorously rinsed them with sea water followed by a separate sea water bath for one hour. This rinsing procedure was repeated once more before they were returned to the system and allowed to feed for 10 days. At the end of this period, we removed all the soft tissue and submerged the skeletal elements in a sodium hypochlorite (5% household bleach) solution for 24 hours to clean and remove any remaining soft tissue. The skeletal elements were then rinsed with tap water and air-dried. The calcein marks from both individuals were clearly visible on the demipyramids which brightly fluoresced when illuminated with UV light.

On March 14, 2008, we placed each of the 72 replicates of the factorial experiment in individual (independent) beakers with 300 ml of the designated calcein solution: 100%, 50%, or 10%. When each treatment finished its designated soaking time (2, 4, 12, or 24 hours), we removed each replicate from its calcein bath, and gave it two, one-hour rinse cycles, similar to those in the trials. These replicates remained outside of the sea system in new, 300 ml filtered seawater cups, to standardize the conditions between treatments. In addition, after 12 hours we refreshed the calcein solution in all 24h treatments. At the conclusion of the experimental period and rinse cycles, all replicates were returned to the randomly assigned positions in the grid of individual cages suspended in a sea table. As with the trials, we allowed the replicates 10 days to feed and grow. At the end of the experimental period, we euthanized all urchins and processed the skeletal elements with sodium hypochlorite as in the trials.

To analyse the presence and intensity of calcein tags, we viewed one demipyramid from each replicate under a Zeiss Stemi SV11 UV light microscope equipped with a GFP filter cube with an excitation wavelength of 470 nm and an emitter wavelength of 500 nm. We chose the epiphysis process as the standard area for quantifying tag intensity because it is the site of most active growth and the standard measure of assessing growth in studies using fluorochrome methods in echinoids (Table 1). Photographs of all

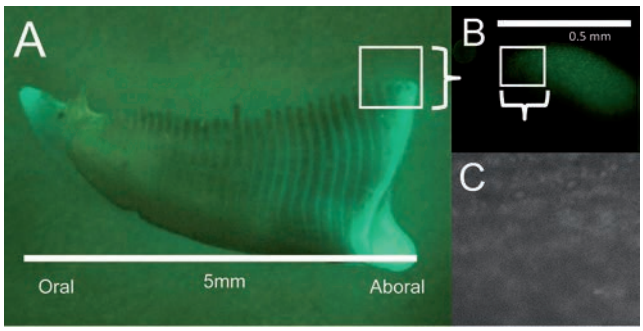

Figure 1. UV illuminated photograph of a single sea urchin demipyramid tagged with calcein. **A.** UV-illuminated demi-pyramid showing calcein tag (fluorescent glow) on the aboral edge. **B.** Epiphysis process. C. Green output of 417^2 pixel selection of epiphysis process used for intensity (gray value) analysis using ImageJ.

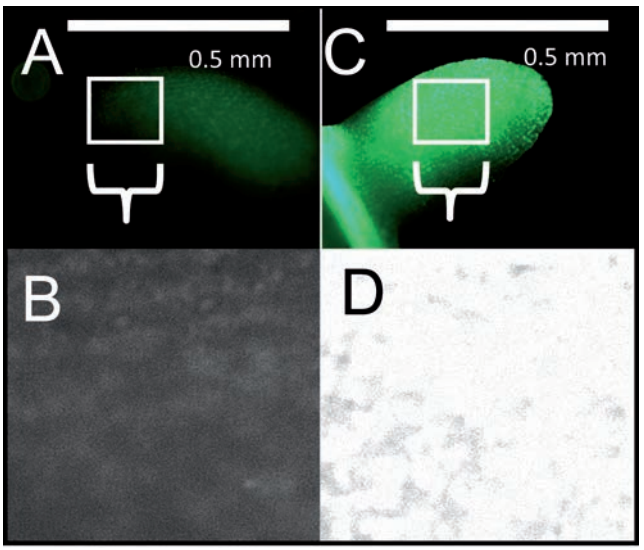

Figure 2. UV illuminated photographs of epiphysis from two sea urchins from different calcein treatments. **A.** Individual from 50% calcein x4 h. **B.** Green output of 417^2 pixel selection of 50% x 4 h (gray value = 78.3). **C.** Individual from 100% calcein x 24 h. **D.** Green output of 417^2 pixel selection of 100% x 24 h (gray value = 248.0).

replicates were taken in a single 8-hour session so that no changes were made to the UV microscope, camera, or settings in Adobe Photoshop (Fig. 1). Using ImageJ software (http://rsbweb.nih.gov/ij), these images were cropped down to 417 x 417 pixel squares of the center of the epiphysis. We extracted the red, green, and blue components of each image using the RGB split tool. This step provided gray-scale versions of the red, green, and blue output of the original image. Because calcein fluoresces green, only the green output was analysed and yielded an intensity measurement in gray values of each (417 x 417) pixel. This dimensionless gray value ranges from 0 to 255 (0 = pure black, 255 = pure white) and measures the brightness of each individual pixel in the image. We used the mean gray value of the pixels for each replicate in the 3 x 4 factorial ANOVA analysis (JMP Version 10.0, SAS Institute Inc).

Results

Our efforts to standardize size among the treatments were successful as there were no significant differences among the 12 concentration x soaking time groups in either diameter ($F_{11,60} = 0.0159$, $p > 0.99$; mean ± SD = 14.95 ± 2.00 mm) or weight ($F_{11,60} = 0.1160$, $p > 0.99$; mean ± SD = 1.63 ± 0.58 g). There were also no mortalities of any urchins during the experiment.

Visual inspection of the calcein-tags clearly showed that sea urchins subjected to longer soaking times and stronger concentrations were brightly tagged, whereas those at shorter soaking times and lower concentrations appeared weakly tagged (Fig. 2). No replicate, either from the control or treatment groups, produced an absolute 0 gray-scale value. The minimum observed in calcein exposures was 7 in the 10% x 2 h treatment. This lowest concentration and shortest exposure had maximum = 34.6 and mean ± SD = 15.4 ± 10.3 gray-scale values. Controls that were not exposed to calcein produced almost completely black images and yielded gray-scale values ranging from 5.5-19.6 (mean ± SD = 9.27 ± 5.5).

The 3 x 4-factorial ANOVA (Table 2) revealed that both duration of exposure and concentration had consistent effects on fluorescence intensity and that there was an interaction between the factors ($F_{6,60} = 3.51$, $p = 0.005$, Fig. 3). Tukey's HSD showed that some treatment groups (100% at 12 h, 50% at 24 h, and 50% at 12 h) were not significantly different from the standard treatment (100% at 24h) although the 50% at 12 h had a much higher variance. Both 50% concentration levels under 12 h yielded significantly lower fluorescence levels. In addition, a

Table 2. ANOVA table of the 3 x 4 factorial analysis with calcein concentration as one factor with 3 levels (100%, 50% & 10%) and soaking hours as the other factor with 4 levels (24 h, 12 h, 4 h & 2 h).

Source	df	Mean Square	F	p
Hours	3	70.382	33.79	< .0001
Concentration	2	127.388	61.16	< .0001
Hours x Concentration	6	7.318	3.51	.0048
Error	60	2.083	–	–

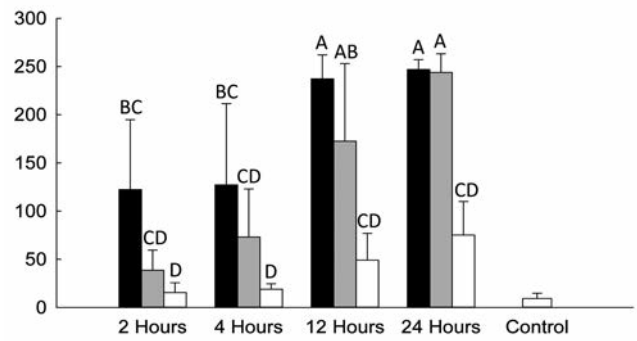

Figure 3. Fluorescence intensity of calcein tag on sea urchin demipyramid, measured as mean gray values (± SD). Sea urchins were assigned to one of twelve groups based on two factors (calcein concentration and soaking time, with three levels for concentration (black = 100%, gray = 50%, white = 10%), and four levels for soaking time (24, 12, 4 & 2 h). Data from untagged control sea urchins are included and provide a background level of "natural" fluorescence. Letters denote significantly different mean fluorescence in treatment groups based on Tukey's HSD.

Table 3. ANOVA table of the Kruskal-Wallis test of the control (no calcein exposure) and 12 other treatment groups.

Source	df	Mean Square	F	p
Treatment Groups	12	2.739	26.59	< 0.001
Error	65	103	–	–

separate nonparametric Kruskal-Wallis analysis (Table 3) and subsequent Tukey's HSD on the 12 calcein-treatment groups plus the control showed that the three groups with the lowest levels of fluorescence were not significantly different from untagged urchins (10% at 2 h, 10% at 4 h, and 50% at 2 h). Finally, the 100% at 2 h (gray value mean = 122.4) was not significantly different from the 100% at 4 h (gray value mean = 127.2) and both were roughly half of the 100% at 24 h (gray value mean = 246.9).

Discussion

The most widely used application of fluorochrome tagging in echinoids is in growth-related studies (Table 1). Russell & Urbaniak (2004) as well as Ellers & Johnson (2009) found short-term effects (reduced growth) of fluorochrome tagging in *Strongylocentrotus droebachiensis*. The former study focused on juveniles < 12 mm test diameter, used the 100% x 24 h protocol, and quantified growth over a 10 week period following tagging. Using reduced concentrations of fluorochrome, reduced soaking times, or some combination, may ameliorate the treatment effects of the procedure and improve this widely-used and effective method.

The mean gray value of the 24 h x100% treatment was 246.9. This high value is near the saturation level (255 = pure white). The 10% concentration level is clearly not strong enough to achieve robust fluorochrome tags even at 24 h (Fig. 3). Although the 50% x 12 h treatment is not significantly different from the 100% x 24 h standard, the mean gray value (172.5) is only 70% of the highest level (246.9) and has the highest variance in either the 12 h or 24 h treatments. However, either the 50% x 24 h (mean gray value = 243.8) or 100% x 12 h (mean gray value = 237.3) are not significantly different from 100% x 24 h level and yielded consistent (low variance) and robust tags (Fig. 3). Therefore, there are no gains in using stronger concentrations at 24 h and the same levels of fluorescence are achieved in half the time (12 h) using the 100% concentration.

Soaking urchins in fluorochrome baths entails isolating individuals in a non-circulating environment. Although it is possible to maintain well-aerated conditions by bubbling air into the bath, metabolic wastes will accumulate during the tagging procedure. We did not - and we know of no other studies that have - measure metabolic waste levels, e.g., ammonia. However, to avoid the potential negative effects these wastes may have on urchins, we recommend using the 100% calcein concentration for 12 h rather than a lower concentration for a longer period, i.e., 100% x 12 h rather than 50% x 24 h.

Our study focused only on small urchins (all < 18.3 mm diameter and < 2.85 g) using the soaking method to apply the fluorochrome and did not address issues relating to injection methods usually employed with larger individuals. For example, in a field study of *S. droebachiensis* Russell et al. (1998) injected urchins > 20 mm test diameter and soaked smaller individuals. Ellers & Johnson (2009) used weight-specific injection doses for both calcein (15 mg kg^{-1}) and tetracycline (50 mg kg^{-1}) which were lower than had previously been used. They found these lower injection doses produced "clear lines" on the demipyramids and their results suggest that a similar study to ours should be conducted to rigorously establish injection protocols.

Finally, we presented a novel approach to quantifying the level of florescence intensity on echinoid skeletal elements using software that is freely available and in the public domain (ImageJ, http://rsbweb.nih.gov/ij). Although the methods we provide can be used for quantifying and comparing fluorescence levels within a particular study, we caution against statistical comparisons between studies. Several factors can affect quantifying the intensity of fluorescence levels on the images and should be standardized to the greatest degree possible. For example, we were careful to obtain all the images during one 8-hour session on the microscope because the efficiency of the UV

system can change with the number of hours logged on the bulb providing the light source. We also made sure that all the settings in the image capture program (Adobe Photoshop) did not change between samples. Different image capture software and the settings within these different programs vary and any differences in these factors could affect quantitative comparisons between studies. One essential component in any study attempting to measure levels of florescence intensity is a control group that is not tagged. Quantifying background levels of "natural" fluorescence will be a key step to standardizing gray-scale measures across studies.

Acknowledgments

We thank Larry Harris, Chris Hill, and Jay Gingrich for supplying the urchins. This research was supported by USDA and NSF grants to MPR. Sam Morelli and the Biology Department of Villanova supplied additional laboratory resources. Martin Fox, Lauren Elliot, and Michelle Harris provided laboratory assistance.

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