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果蠅單眼的發育調控基因之篩選

得獎獎項

大會獎：一等獎

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關鍵字：果蠅、單眼、*ras*

作者簡介

我是庭軒，現在就讀於北一女中數理資優班三年級。

小學的時候，在語文中找到了自信及快樂。

而就在小五那一年，因緣際會參加了「國際小學科學奧林匹亞」，進入了奧秘的科學殿堂。升上國中後，多采多姿的競賽及活動沒有阻擾我對科學的愛好，在國三課業最繁忙的時候，毅然決然地與同學以「翻滾吧！電池車」之作品，一起參與了國中小科學展覽競賽，並得到了台南區第一名。

升上高中後，幸運的考上了北一女中數理資優班，在緊湊的課業之中，秉持著科學的熱忱，兩度代表學校參與在新加坡舉行的「國際科學青年論壇」，不僅大大的拓展了我的世界觀，也結交認識了許多愛好科學的異國朋友。

高二這一年，榮幸進入了孫以瀚教授實驗室，做了一個專題研究。在教授、學長的指導之下，徜徉於研究的領域，在小小的果蠅中，發現大大的驚奇。所以就算高二及高三生活充滿精彩的活動及競賽，我也不忘在放學後及六日去關心我那群小小果蠅們呢。:)

最後，真的很開心能夠參加國際科展，跟大家一起分享我在研究上所獲得的那些感動及喜悅。

摘要

(一)、中文摘要

在果蠅成蟲的頭頂上方三顆可以感光的單眼(ocelli)，其構造和複眼有顯著的差異，但單眼及複眼在果蠅幼蟲時期，皆從一片特別的組織 eye-antenna disc 發育而來。而且目前已知會表現在單眼的一些特定基因，也是會調控複眼發育的基因，如 *sine oculis (so)*，*eyes absent (eya)* 等。但到底是哪些特定的基因決定了單眼與複眼的差異，目前並不是很清楚，因此我想探討果蠅單眼細胞在發育過程中，有哪些特定的基因會調控果蠅單眼的生成與變化。

本實驗主要是利用果蠅研究上常使用的基因表現系統(UAS-GAL4 system)進行研究，我們挑選許多和複眼發育相關的基因、胚胎生長發育相關基因進行表現，觀察其單眼性狀有無變化，篩選出會影響單眼發育的相關基因後，再進一步研究此基因的可能作用機制。

從實驗的結果中發現當表現 *ras* 及 *Egfr* 的顯性抑制型 (ras^{DN}, $Egfr^{DN}$)時，在掃描式電子顯微鏡下可觀察到三顆單眼會明顯變小。而在幼蟲時期的 eye-antenna disc，利用免疫螢光染色以及雷射共軛焦顯微鏡觀察到當表現 ras^{DN}、$Egfr^{DN}$ 時, 單眼標記基因(marker gene) Eyes absent (Eya)表現的強度顯著降低。再加上已知 ras 為 Egfr 的下游基因，綜合這些結果，我們認為 Egfr-Ras 以及其下游基因的訊號傳遞對於單眼發育是很重要的。

另外在視網膜電流圖（electroretinogram）的實驗當中，發現 Egfr, ras 訊息傳遞被抑制的果蠅單眼在對光線刺激的反應強度比起正常的果蠅下降許多，因此可推論 Egfr, ras 的訊息傳遞不僅會影響單眼的發育，也會影響單眼的正常感光的功能。

(二)、英文摘要

The visual system in *Drosophila* is composed of two large compound eyes and three simple eyes (ocelli). Since the compound eye and ocelli originates from the same eye-antenna disc, I am interested in what is the main factor that controls the ifferences between the compound eye and the ocelli. I would like to screen the genes involved in compound eye/ocelli cell fate determination and also genes that play significant roles in ocellar development. The research is conducted by using UAS/ GAL4 system as the method to express or knockdown specific genes, singly or in combination. I use trol-Gal4 to express genes in the ocellar precursor cells during the Drosophila development. The adult ocellar phenotype and ocellar marker gene expression pattern can be observed by microscopy and confocal microscopy respectively. So far, I found that expression of several genes in the ocelli can affect the ocellar development. The obvious phenotype comes from the inhibition of EGFR or ras signaling in the ocellar precursor cells. We can found much smaller ocelli in the flies that overexpression of dominant negative form of ras (ras^{DN}) or the Egfr dominant negative form ($Egfr^{DN}$) in the ocellar precursor cells. These smaller ocelli could be easily observed by high-resolution scanning electron microscopy, but not light microscopy. In addition, ocellar marker Eyes absent (Eya) also reduce dramatically in the ocellar precursor cells of these larvae. These suggest that EGFR or RAS signaling indeed affect the ocelli from the larval development. It has been shown that ras is a signaling component downstream of Egfr. Together these data, I suggest that EGFR-RAS and downstream signaling pathway control the ocellar development. Furthermore, we found the ocellar mutant flies show dramatically weaker electroretinogram (ERG) response to light stimulate. These suggest that EGFR-RAS signaling not only affect ocellar development, but also normal ocellar function.

目錄

一、前言

(一)研究動機

在果蠅成蟲的頭頂上方三顆可以感光的單眼(ocelli)，其構造和複眼有顯著的差異，但是單眼以及複眼在果蠅幼蟲時期皆從一片特別的組織 eye-antenna disc 發育而來. 而且目前已知會表現在單眼的一些特定基因，也會參與調控複眼的發育，如 *sine oculis (so), eyes absent (eya)* 等. 目前並不清楚有哪些基因參與單眼生長的調控，因此我想研究有哪些基因參與及調控果蠅單眼的發育?另外也十分好奇是否有基因能造成單眼轉化為複眼。

(二) 、研究目的

希望利用 UAS-Gal4 system 來快速篩選會影響果蠅 ocelli 發育的基因，並希望能找出是否有基因能夠造成單眼轉化成複眼。

二、研究過程或方法

(一)、背景介紹

1、果蠅視覺系統介紹

果蠅的視覺系統主要由三顆單眼及兩顆複眼構成，其排列方式如下圖：

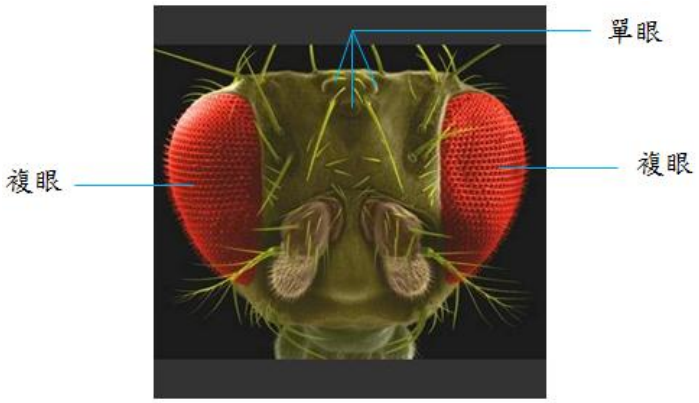
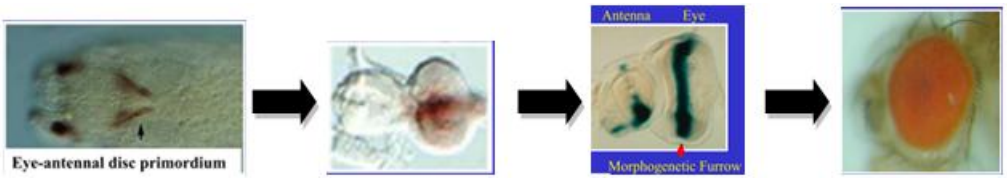
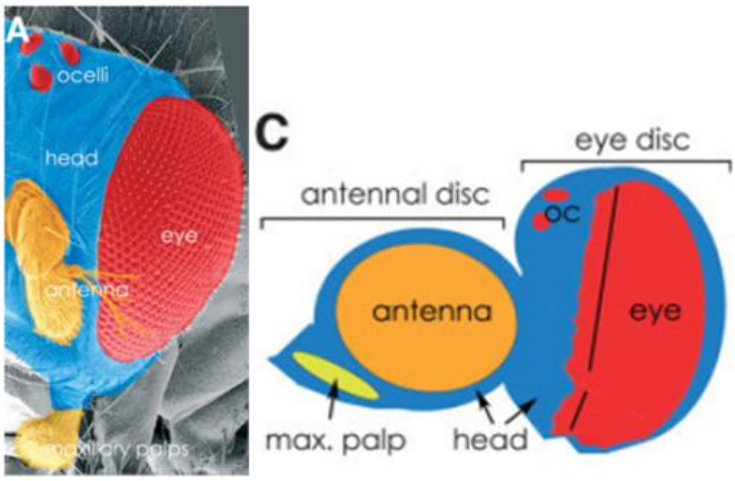


Fig. 1 果蠅的視覺系統-包含複眼及三顆單眼

而其視覺系統是由一片 eye-antenna disc 發育而來的，其在從幼蟲(larva)發育到成蟲中，此片 disc 會逐漸發育成視覺系統以及觸角。如下圖：



而令人感到十分有興趣的是，在同一片組織上(eye-antenna disc)，細胞發育的機制卻十分不同，在同一片組織的不同的位置，各自會發育成單眼及複眼。以下的圖為不同位置發育出不同視覺構造的示意圖: (不同的顏色即代表不同組織)



(*Developmental dynamics* 232(3), 673-84.)

Fig. 2 單眼和複眼發源於同一片原生基(eye-antenna disc)

單眼是一種只有單個晶狀體的眼睛構造，單眼中的「單」不是就細胞數量或是感光精確度而言，事實上這種感光的構造通常是十分複雜的，而之所以被命名為單眼，是因為其只有一個鏡頭(晶狀體)。動物的眼睛構造通常和所棲息的環境以及所必需進行的求生行為有關，節肢動物在各種不同的生存條件下(例如棲息地的不同、需具備不同的感光構造以找到食物跟同類等)，各自演化出不同種的感光構造以適應環境。

單眼有分兩種不同的形態一為單眼，另一為側單眼，由於果蠅屬於單眼的分類，以下僅介紹單眼的特徵。

單眼在很多昆蟲上都找得到，但是構造以及功能各異。飛行昆蟲的單眼發育較良好、顯著 (如蜜蜂蜻蜓還有蝗蟲)，通常是三個。部分陸生昆蟲中間的單眼會退化，只剩兩個(例如螞蟻及蟑螂)。

單眼有兩個特徵，一般昆蟲的單眼皆保有此二特徵。(1)單個鏡頭的折射力通常不足以在感光層形成圖像。(2)單眼從第一階的感光器到第二級神經元有大量的收斂率。這兩個因素導致單眼無法感知圖像，只具感光功能。由於大光圈、低焦距比的鏡頭、高收斂率和突觸收益，單眼通常被認為是比複眼對光線更為敏感的感光構造。此外，單眼的視神經神經排列較小眼複雜，且具備較大的直徑（經常在動物的神經系統是最大的直徑神經元），因此，單眼通常被認為比複眼擁有更快的感光能力。

一個常見有關單眼的應用理論是鑑於其欠焦的性質、寬領域的視野、很高的收集光之能力，它們能被用來協助維持飛行的穩定，目前已藉由蝗蟲感光修正飛行和蜻蜓的限制範圍飛行被證明。

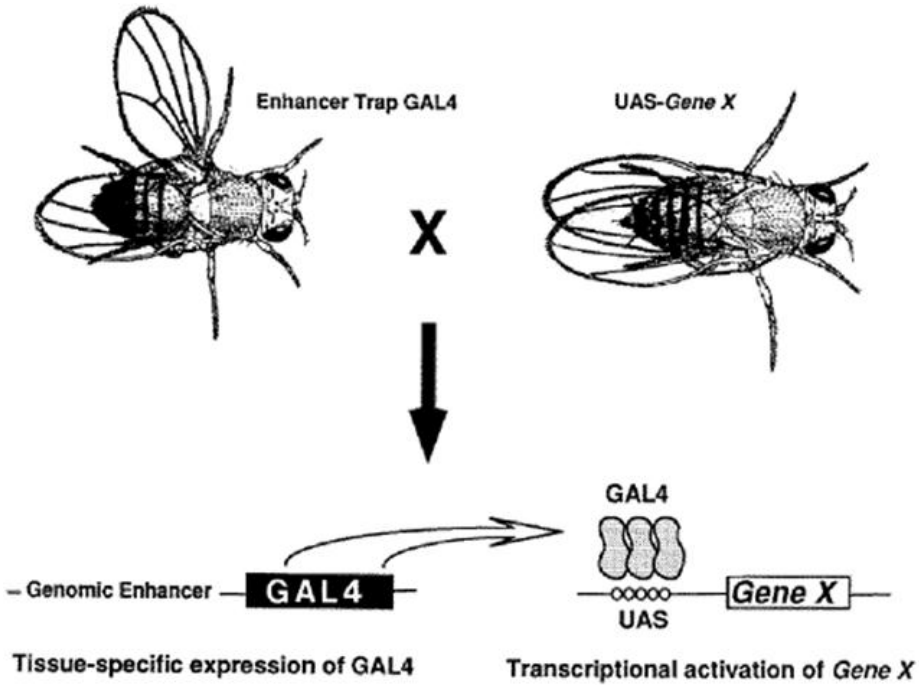
雖然單眼長久以來被認為因為有欠焦的性質而無法感知圖像，但最近的研究也發現，有些昆蟲的單眼（最顯著的為蜻蜓，但也有一些小蜂）有能力在單眼的鏡頭上，或是接近感光層的位置形成圖像。進一步的研究表明，這些眼睛不僅感測視覺，他們也決定動作。當接受到紫外光的刺激後，二階神經元的蜻蜓中間單眼對於移動中的欄杆以及光柵上部的感光反應較下部強烈許多。蜻蜓的單眼是發達、專門的視覺器官,它目前被認為可以支持一些蜻蜓的特殊飛行技巧。

看起來似乎單眼對於昆蟲有相當重要的功能, 而且在昆蟲的演化上,單眼是相當保守的構造。然而我們對於單眼的發育過程並不瞭解，因此在本研究中，我們利用果蠅作為一個模式生物，並利用 UAS-Gal4 System 來篩選會影響單眼發育的基因。

2、UAS-GAL4 system

有許多方法可以調控基因的表現以及了解其功能，在果蠅的遺傳研究模式中，常常使用大規模突變性狀的篩選來了解基因功能，本實驗室使用 UAS-GAL4 system 作為調控基因表現之方法。 我們運用 UAS-GAL4 system 來大量篩選會影響果蠅單眼性狀的基因，目前篩選的基因包括已知會影響果蠅複眼發育的基因即會影響果蠅胚胎發育的基因。

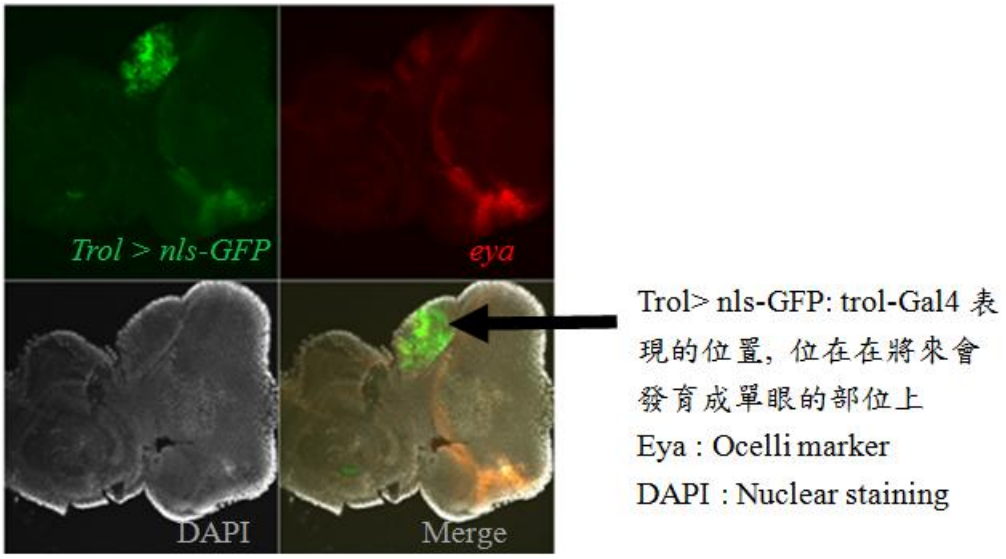
UAS-GAL4 system 的實驗原理，是利用基因轉殖技術，使即將交配的雌雄果蠅，其一帶有 endogenous promoter/enhancer 和 GAL4 基因，另一帶有 UAS(GAL4 binding site)和欲研究的基因。兩者交配後的子代，其 GAL4 在和 UAS 結合後，可使欲研究的基因表現。



(*Development 118(2), 401-15*)

Fig. 3 UAS-GAL4 系統被用來表現我們欲篩選的基因

首先為了確認我們所使用的 trol-Gal4 表現的位置正確，我利用 trol-Gal4 > nls-GFP(核內表現的綠色螢光蛋白)，並做免疫染色來觀察基因表現位置，發現基因的確在將來會發育成單眼的位置上表現。由此可知 trol-Gal4 的 genotype 表現位置和文獻中所記載的結果相符合 (Trol-Gal4 即 NP4065-Gal4, 記載於 Developmental Biology (2010) 337 , 246–258)。於是接下來即可利用 GAL4-UAS systam 來進行單眼發育調控基因之篩選。以下為 GAL4>nls-GFP 免疫染色後，於雷射共焦顯微鏡下的照片：



(二)、研究方法

1、實驗流程

在我的實驗中，使用 trol-GAL4(x) 處女蠅 (以下將以此符號表示處女蠅)與帶有 UAS 的公果蠅以及所連接之基因交配時，GAL4 誘使 UAS 下游基因的表現。先利用各種 UAS-基因品系的雄性果蠅與 trol-GAL4 的處女蠅交配之後(雄蟲及雌蟲皆為五隻)，觀察子代成蟲的性狀。

我們藉由下圖的方式，配合 balancer 的功能協助基因表現在正確的位置上，觀察正確表現 UAS 下游基因的子代成蟲。

剔除掉表現 balancer 的果蠅後，我利用果蠅觀察台以及解剖顯微鏡觀察果蠅單眼的性狀。將單眼有變化的果蠅個別挑出來，放置於另一附有照相設備的解剖顯微鏡下拍攝其單眼變化。另外我們會再利用此方法，讓帶有可能調控單眼變化的基因之果蠅交配數次，計算比例並且觀察每次性狀變化。

再者為了拍攝更精確的單眼性狀變化照片，我們利用了 Anette Brockmann, Marı´a A. Domı´nguez-Cejudo, Gabriele Amore, and Fernando Casares1 (2011) Developmental dynamics 240:75–85 所記錄的單眼切片方式，用刀片把單眼所在的那片組織切片，然後放於光學顯微鏡下拍照。以下的實驗結果如用此方式會標示在圖上方，目前應用此方式的皆為以前有變化的果蠅，我再多次使他們交配，以拍攝更為清晰的照片。

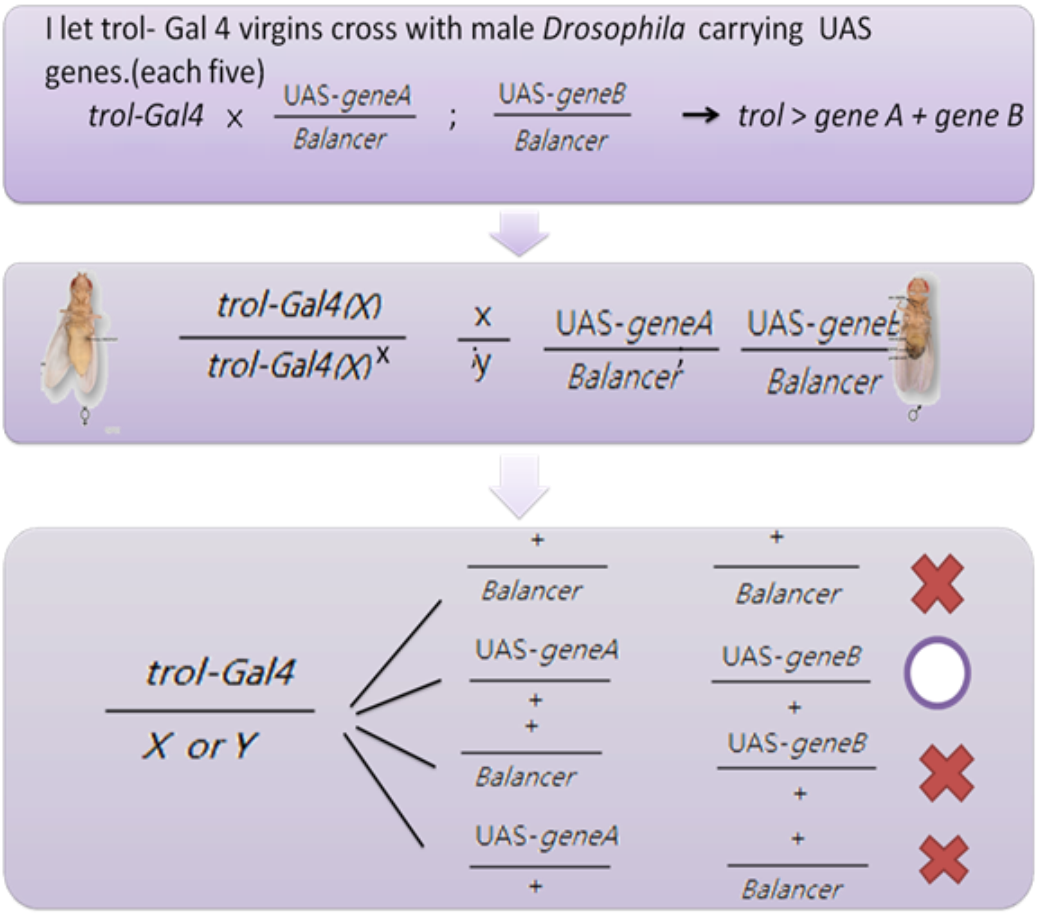


Fig. 4 果蠅的篩選步驟

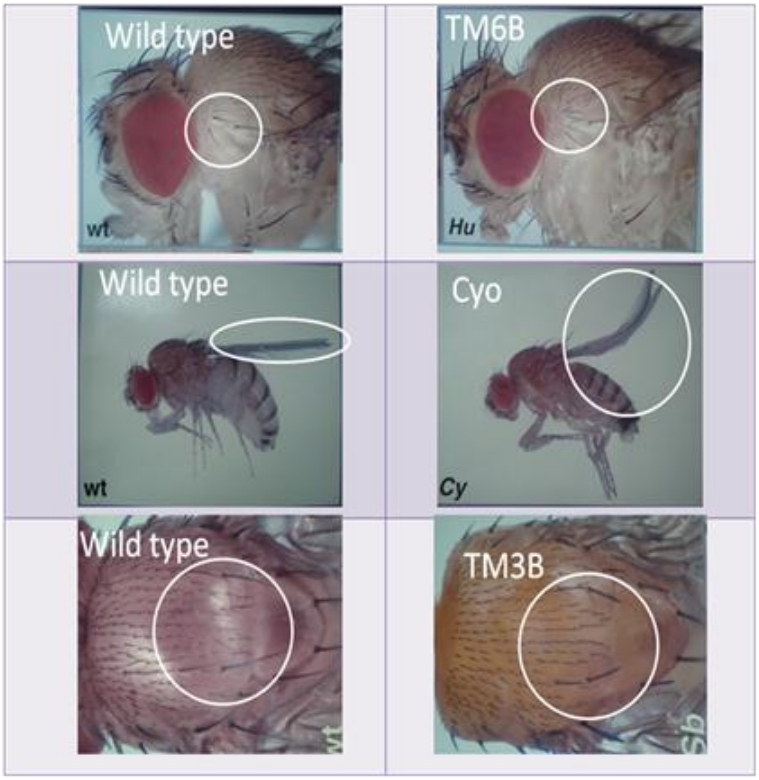


Fig. 5 各種不同的 dominant marker

2、免疫螢光染色:

在我初步得知哪些基因有調控單眼變化的現象後，會解剖三齡幼蟲，來觀察其在三齡時，在 eye- antenna disc 中單眼的發育如何從複眼中區隔開來。我們會使用免疫螢光染色來追蹤目標基因所產生的蛋白質。以下僅介紹免疫染色的原理：

免疫染色是利用免疫學的原理與細胞化學結合而成的技術。根據抗原跟與抗體不同特異性結合的特點，檢測細胞內某種特定蛋白質及膜表面抗原和受體等大分子的存在以及分布。蛋白質種類十分繁多，均具有抗原性，因此當將人或動物的某種蛋白質做為抗原注入另一種生物體內，則產生與抗原相對的特異性抗體(免疫球蛋白)，將此特異性抗體從血清中提出結合上某種標記物即成標記抗體。

在免疫染色中標記抗體與抗原的結合法主要分為兩種，一為直接法：用標記抗體與抗體中的相應抗原直接結合，操作較簡易、特異性高但敏感性較差，此法較適合用於檢測未知抗原。另一種為間接法 (我的實驗採用此法)：先用未標記的、具有特異性的第一抗體與欲檢測細胞中的相應抗原結合，然後再以標記的第二抗體與特異性的第一抗體結合，第二抗體為利用第一抗體作為抗原，注入另一動物體內所誘導產生的抗體，然後再結合以標記物。通過這樣的放大作用，使抗原上的標記物大大增多，故間接法較直接法的敏感性增高許多。

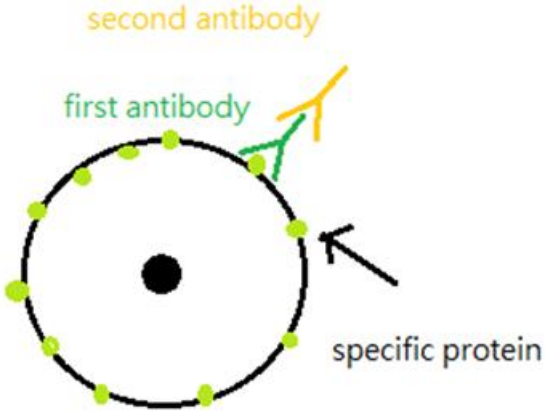


Fig. 6 免疫染色之示意圖

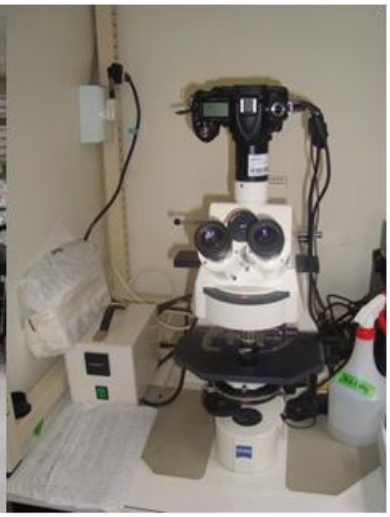
(三) 、研究設備及器材

研究設備為基因轉殖的果蠅以及光學顯微鏡、解剖顯微鏡，照相設備，共焦螢光顯微鏡，培養管。研究材料有：PBS，Paraformalehyde，PBST，Glycerol。

果蠅培養箱(10*10)

果蠅觀察台

解剖顯微鏡(附照相設備)

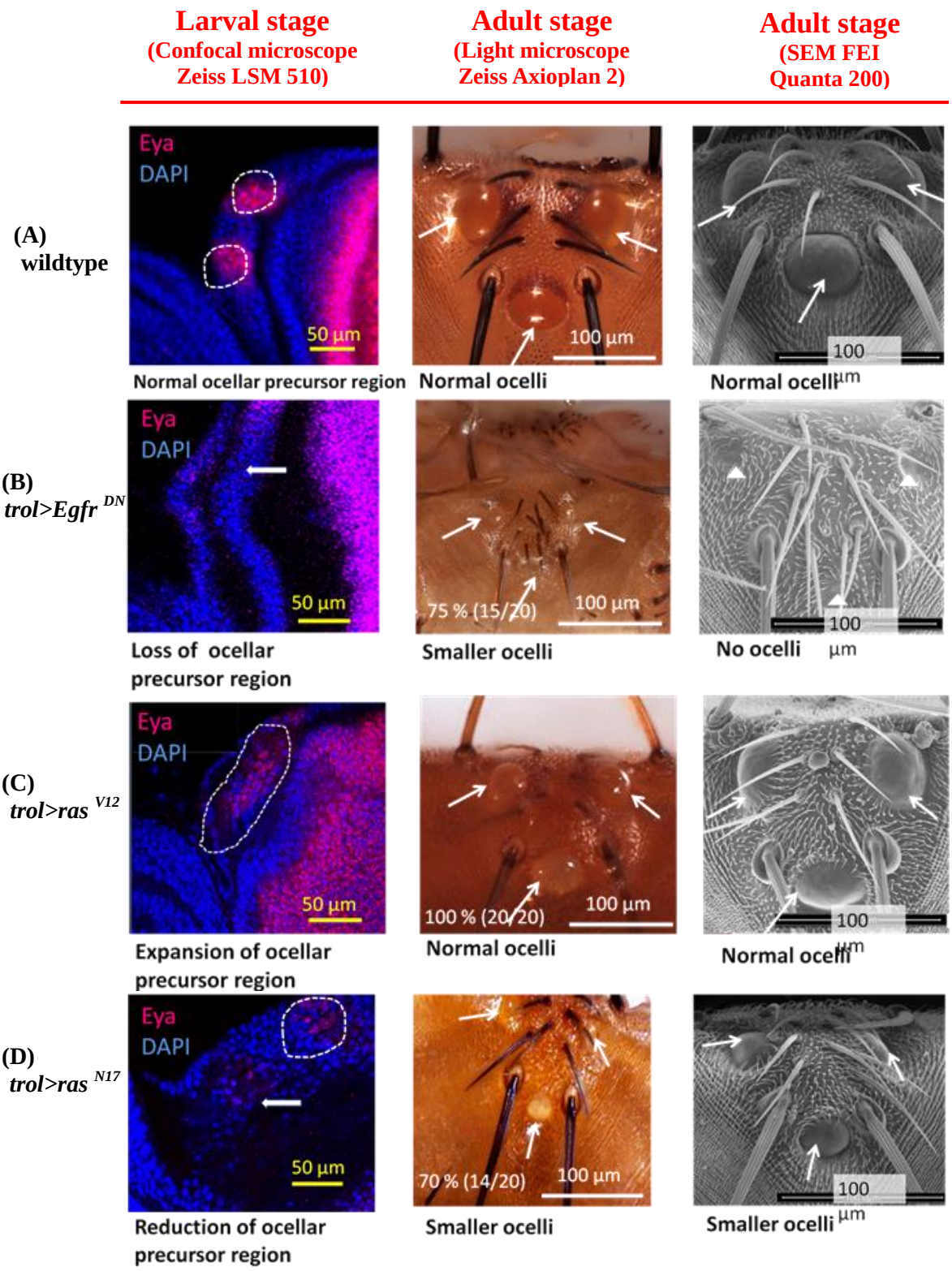


光學顯微鏡(附照相設備)

Fig. 7 實驗器材

三、研究結果與討論

(一) 、研究結果



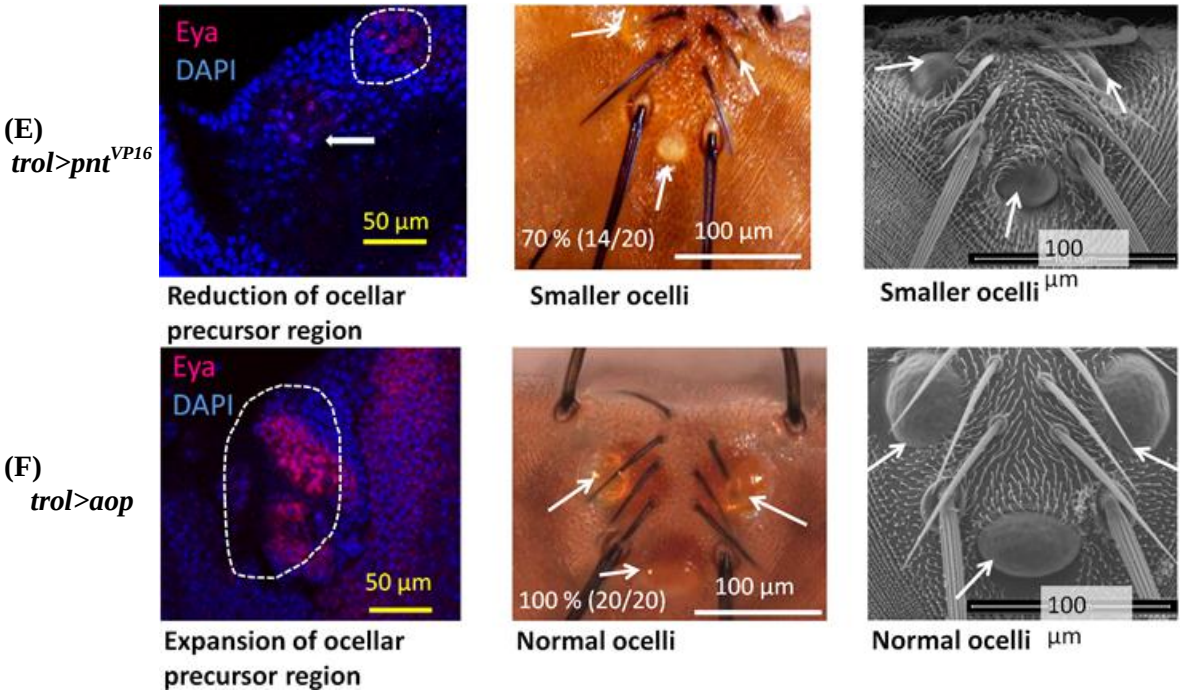


Fig. 8 Expression of *eya* in eye-antenna disc and eye morphology in adult flies

(A) Wild type

(B) *trol>Egfr*^{DN}, a dominant negative form of *Egfr* :
In larval stage, it shows the loss of ocellar region. In adult *Drosophila*, Some of the mutant flies' ocelli are much smaller, while the others' ocelli seemed to be missing.

(C) *trol>ras*^{V12}, a constitutive active form of *ras* :
It exhibits expanded Eya staining area in eye-antenna disc, and normal ocelli in adult.

(D) *trol>ras*^{N17}, a dominant negative form of *ras* :
In larval stage, it demonstrates the expanded Eya staining area. In adult Drosophila, three ocelli are present but decrease dramatically in their size under SEM.
(The diameter of the medial ocellus dropped from 46.4 µm to 28.6 µm.)

(E) *trol> pointed*^{VP16}, a constitutive active form of *pointed* :
It shows expanded Eya staining area in eye-antenna disc, and normal ocelli in adult.

(F) *trol>aop*, an inhibitory transcriptional factor
It exhibits loss of Eya staining area in eye-antenna disc, and normal ocelli in adult.

(C) Electroretinogram (ERG) response to light stimulation of ocelli

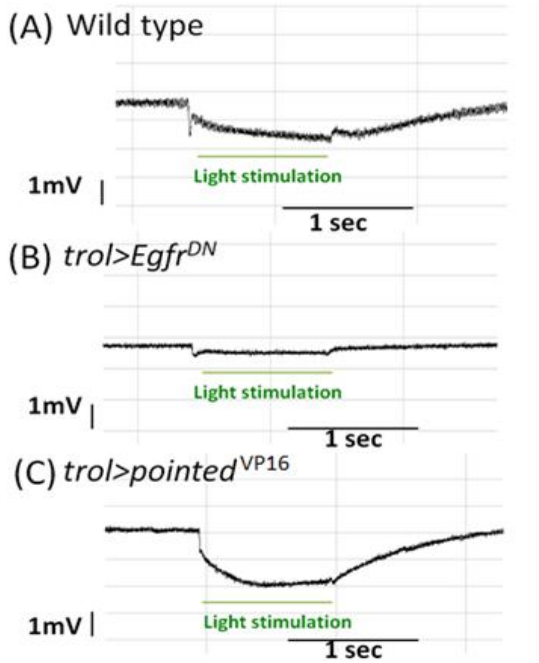


Fig. 9 The Electroretinogram (ERG) response of ocelli in
(A) Wild type
(B) ocellar mutant (*trol>Egfr*^{DN})
(C) Ocellar mutant *(trol>pointed*^{VP16}*)*

The ERG is measured by the voltage difference between an active electrode and a ground electrode. The results showed that compared to ocelli of wild type, the mutant ocelli of *trol>Egfr*^{DN} showed little or no voltage change in response to light stimulation. In contrast, the mutant ocelli of *trol>pointed*^{VP16} demonstrated stronger voltage change in response to light stimulation.

(D) Electroretinogram (ERG) response to light stimulation of compund eyes

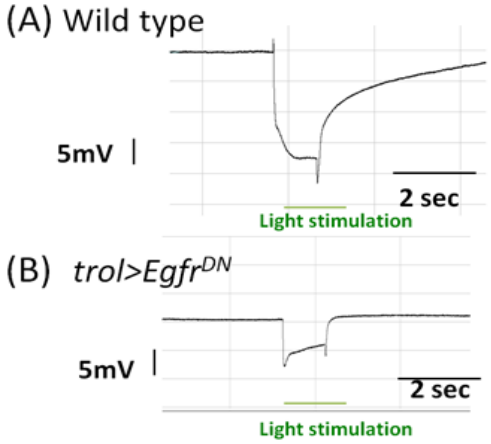


Fig. 10 The Electroretinogram (ERG) response of compound eyes in
(A) Wild type
(B) Ocellar mutant (*trol>Egfr* ^{DN})

The ERG of ocellar mutant's compund eyes was measured to see if the mutant ocelli would influence the ERG response of compound eyes. The result showed that the ocellar mutant exhibited smaller voltage amplitude.

(E) Results of overexpression of *eyg+hth*

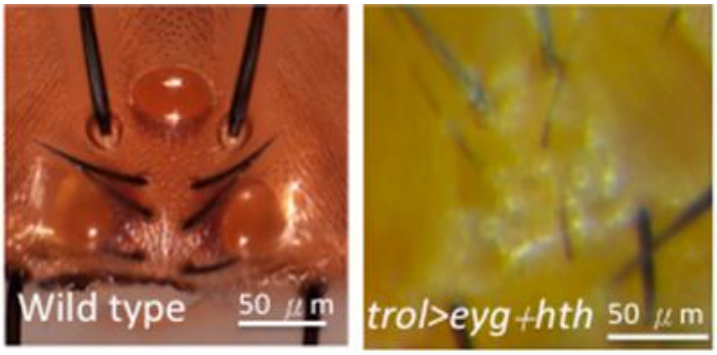


Fig. 11 The phenotype change of ocelli when co-expressing *eyg* and *hth*
Three ocelli turn into several small ocelli

(F) Results of overexpression of *eyg+ser*

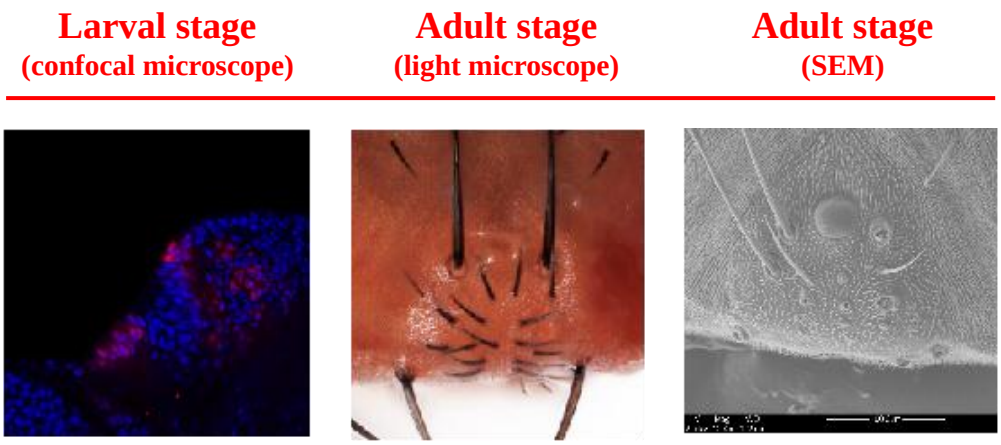


Fig. 12 The phenotype change of ocelli when overexpressing e*yg+ser*
Some of the mutants' only own the medial ocellus, while some of the mutants have three ocelli but except the medial ocellus, other two lateral ocelli were dramatically reduced in size.

(二)、研究討論

在本實驗中，我們利用了果蠅作為模式生物篩選出了幾個基因可能會對於單眼的發育會造成影響的，包含 *eyg-1 + hth*, *eya + so*, *ras* 等基因，其中 *eya* 和 *so* 是原本就會表現在單眼的標記基因，而同時表現這兩者基因，卻能造成各種不同的 phenotype，包含融合、多顆小單眼增生、單眼形狀改變等等，推測可能是因為這兩者基因的表現，造成了原本單眼發育的領域擴張，形成融合的大型單眼 (trol-gal4 表現的位置比原本單眼發育的位置更大) ，或著是造成更多小型單眼被發育出來，而這其中的差異目前還不是很清楚。不過表現單眼標記基因 *eya + so* 會造成單眼性狀上的改變，也進一步確認我們所採用的實驗系統是有效的。

在大量表現 *eyg-1 + hth* 的部份，造成了子代單眼的 phenotype 為單眼成倒 V 形狀，且增生了多個感光細胞，另外其複眼沒有發生任何變化，推測是因為 *hth* 藉由抑制 *MF-initiation*(會造成複眼的發育)來抑制了複眼的發育，但是 *eyg-1* 可能透過抑制 wg 造成 *MF-initiation* 的啟動(如圖 11)，所以可能兩個所對複眼造成的功用相抵消了，也因此當

我們共同表現了 *eyg-1 + hth* 時，複眼沒有發生改變。但是值得注意的事 eyg 有另外的功能是造成細胞的增生，也因此我們推論可能是由於此功能造成所表現的 phenotype 有感光細胞增生現象。

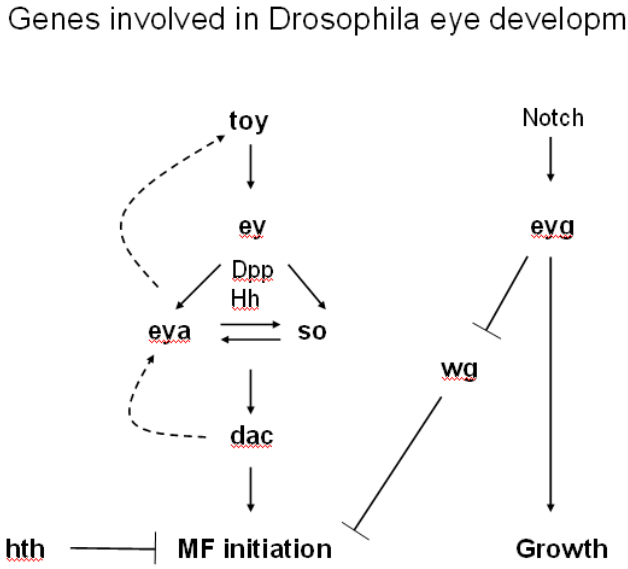


Fig. 13 *eyg* 跟 *hth* 作用機制之探討

而在大量表現 *ras* 基因的部份，則得到了非常有趣的結果。在 *trol* > *ras* 的情況下，會發現到總是在頭部後方相同的位置多出一顆單眼細胞，而且原本在中間前面的那顆單眼也會移到後方。這是沒有預期到的現象。中間偏前方的單眼原本由兩片 eye-disc 單眼 primordium 融合而成， 是最特殊的一顆， 而 *ras* 基因為控制生長的重要基因，從其他研究得知啟動 *ras* 會造成細胞的增生，而抑制 ras 則會造成細胞生長速率的減緩。但在我們的實驗中卻發現到了 *trol* > *ras* 會造成原本該融合的兩顆單眼細胞變成不融合了，而且其位置由前方變到後方。這是非常有趣的現象，值得再進一步的深入研究，因為似乎這是第一次發現到能將兩片 disc 融合而成的單眼分開成兩個的實驗。

接著當我們利用 *trol>ras*^{N17} 時候，我們發現原本的三顆單眼皆消失不見了，*ras*^{N17} 為 *ras* 基因的顯性抑制(dominant negative)型式，意指當表現此基因時，細胞中會同時帶有野生型以及突變型的蛋白質，突變型的蛋白質會抑制本來蛋白質的功能，而呈現顯性抑制的現象。也因此此基因可以說是抑制了 *ras* 基因的功能，而其也抑制單眼的生成。另外當我們表現另外一種形式的 *ras*^{N17} */Cyo(II)* (此種基因型表現的強度並沒有前者強)，還是抑制了單眼的形成，但是單眼還剩下兩顆。從以上的數據我們可以推估 *ras* 的確在單眼發遇上扮演了十分重要的角色，而且 *ras* 表現程度的強弱會顯著地影響單眼的發育。

在知道 *ras* 扮演了如此重要的角色後，我們便想找出 ras 的上下游基因，進而建構出單眼發育的調控路徑，而我們也發現當表現了的顯性抑制型，我們得到了與表現 *ras* 的顯性抑制型相同的結果。在過去的文獻中，我們發現 *ras* 是 *Egfr* 的下游基因之一(如圖 12)，也因此我們推測 *Egfr* 在單眼發育的過程，扮演了啟動 *ras* 基因的角色。而我們建構了單眼發育調控路徑

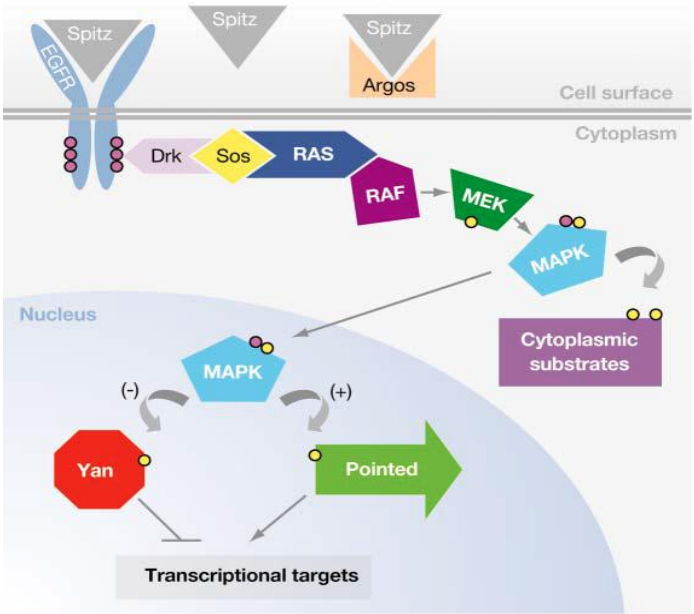


Fig. 14 *ras* 是 *Egfr* 的下游基因之一
(Pavithra Vivekanand and Ilaria Rebay(2006),
The Annual Review of Genetics)

Fig. 15 單眼發育調控路徑

四、結論與應用

(一)、結論

目前已觀察到同時在單眼部位表現 *eyg* 和 *hth*； *eya* 和 *so*； *tsh* 和 $N^{[act]}$； *Dll* 和 *hth* 時，單眼性狀皆會發生改變。而目前推測影響單眼發育最重要的基因為 *ras* ,表現 *ras* 顯性抑制型的基因時，單眼會消失不見。除此之外，我們也找到 *ras* 的上游基因 *Egfr* 及下游基因 *pointed*，因此我們建構出單眼發育的可能調控路徑為：

***Egf* → *Egfr* → *ras* → *pointed* → ocellar development**

五、參考資料及其他

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A. Introduction

(A) Motivation

The *Drosophila* adult visual system consists of three ocelli located on the top of the head, and two large compound eyes located at the side of the head. What intrigues me is that both light-sensing organs arise from the same progenitor cells (eye-antenna disc) during the third instar larva (Fig. 1), but have very distinct features. The molecular mechanism for compound eye development is well studied. I would like to identify the main genes and mechanisms that control the development of ocelli.

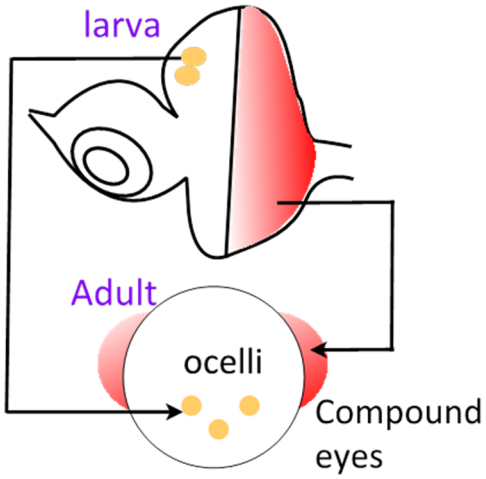


Fig. 1 ocelli and compound eyes arise from the same eye-antenna disc

(B) Background Analysis and Theory

1. The Drosophila visual system

The adult *Drosophila* head has two large compound eyes and three ocelli (simple eyes) (Fig. 2). The three ocelli, one anterior (or medial) ocellus and two posterior (or lateral) ocelli, are located at the vertices of a triangular patch of cuticle on the head vertex (Fig.2). The head vertex is the dorsal region that separates the two compound eyes and is formed from the fusion of the two eye-antennal discs (Haynie and Bryant, 1986). The compound eyes and ocelli are surrounded by specific bristles.

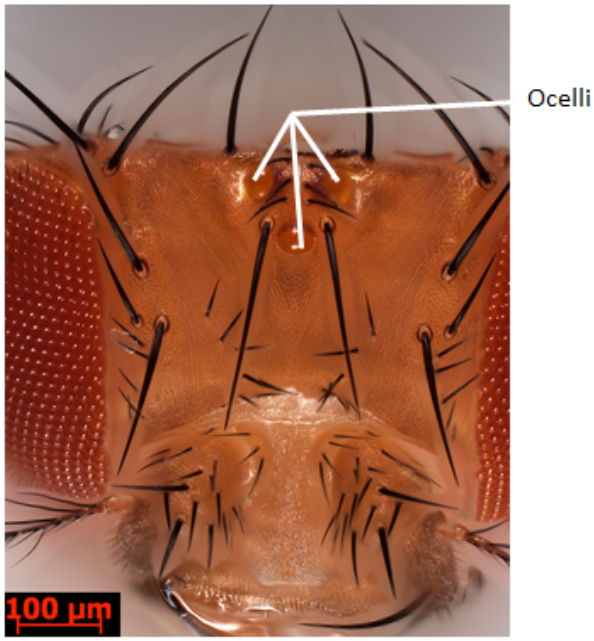
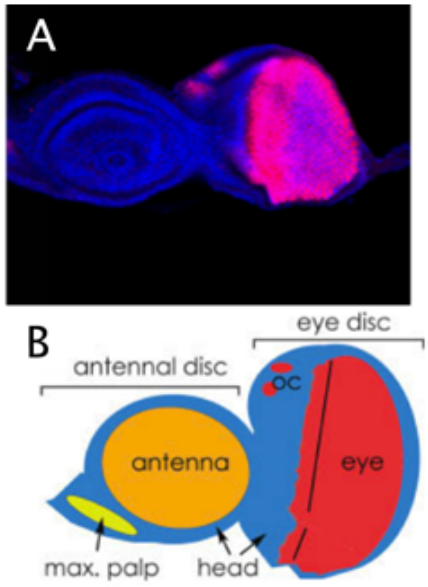


Fig. 2 The adult compound eyes and ocelli

What's intriguing is that cells at different places in the same tissue (eye-antenna disc) will develop into ocelli and compound eyes separately (Fig.3).



(*Developmental dynamics* 232(3),673-84.)

Fig. 3 The eye-antenna disc of Drosophila

The adult head of *Drosophila* develops mostly from a pair of eye–antennal discs. **A :** A third-instar eye–antennal disc is doubly labeled with DAPI (stains DNA, in blue) and antibody against Eyes absent protein (Eya, in red). **B:** The

antenna primordia (in orange) is shown by the expression of Distalless (Dll) at this stage. In yellow is the region of the disc fate mapped to become the maxillary palp.

Ocellus refers to a type of eye that contains single lenses. One common theory of ocellar function is that they help maintain fight stability, due to their high light collective ability and wide range of view (Schuppe and Hengstenberg, 1993). Therefore it would help to know the developmental genes of ocelli if we would like to apply it in other fields.

A number of genes are known to be involved in the development of the ocelli, including *eyes absent (eya)*, *sine oculis (so)*, *orthodenticle (otd)*, *hedgehog (hh)* and its downstream components *cubitus interuptus (ci)* and *fused (fu)*),, *EgfR* and its ligand *vein (vn)*, *Notch (N)* pathway (see Wang et al., 2009), and *twin of eyeless (toy)* (Brockmann et al., 2010). The main goal of my research is to identify more genes that are crucial for ocellar development.

2. UAS-GAL4 system:

The overexpression method I used is the UAS-GAL4 system (Brand and Perrimon, 1993). GAL4 is a transcription factor from yeast and is inserted into the fly chromosome. The GAL4 expression timing and pattern can be regulated by the adjacent enhancer/promoter element. Dr. Sun's laboratory has many specific GAL4 drivers which can express in different tissues. GAL4s will bind to the Upstream Activation Sequence (UAS), a transcription factor binding site, which only responds to GAL4. The GAL4-UAS binding will activate the expression of the gene fused to UAS. I can then express different genes in specific tissues by using different GAL4/UAS-transgenes combinations (Fig. 4).

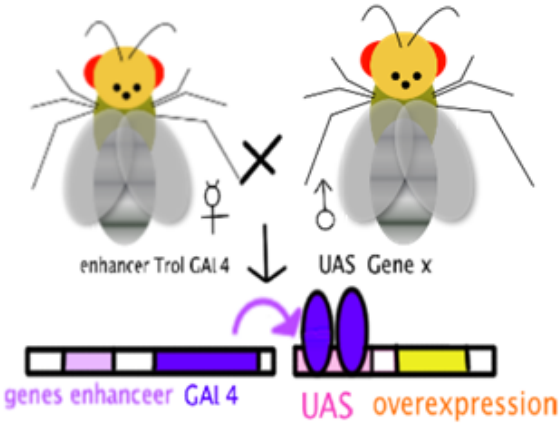


Fig. 4 UAS-Gal4 system was used to overexpress various genes

When GAL4 expression is induced by specific promoter, it will bind to UAS and induce the expression of the gene next to UAS. The *trol-GAL4* is known to be expressed in the ocellar precursor region (*NP4065-GAL4* in Wang et al., 2010). The expression pattern of *trol-GAL4* can be visualized by driving the expression of *UAS-nls-GFP*(the *green fluorescent protein* GFP fused with nucleus localization signal) (abbreviated as *trol>nls-GFP*). In the eye-antenna disc, GFP (green) expression covers the ocellar region. The ocellar region is also marked by the ocellar marker Eya (red) (Fig. 5A arrows), which is also expressed in the developing compound eye.

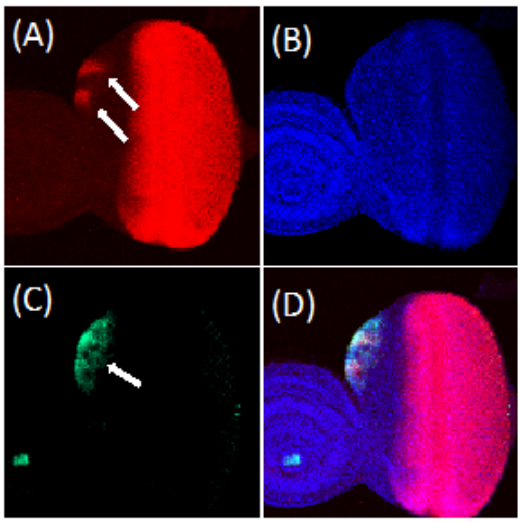


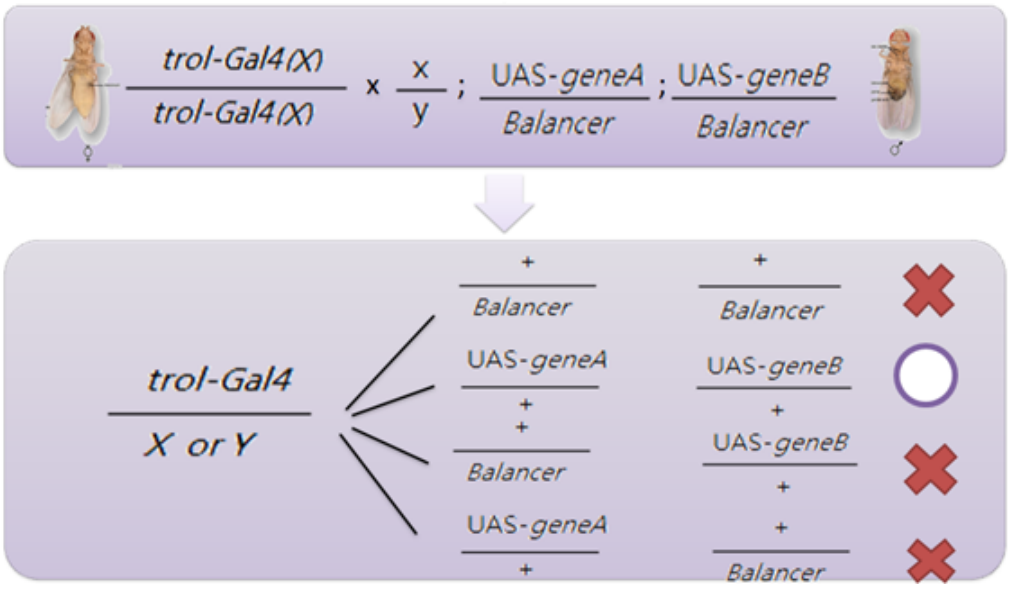
Fig. 5 trol>nls-GFP (trol-GAL4 drives green fluorescence protein)
The place where Trol-Gal4 expresses is the place where ocelli originates.
(A) immunostaining of Eyes absent (Eya)
(B) Eye-antenna disc stained with DAPI (nuclear staining)
(C) nls- GFP signals cover the ocellar region
(D) Merge photo of (A)(B)(C)

B. Experimental methods

(A) The UAS-GAL4 method for targeted gene expression

The *trol-GAL4* virgin Drosophila was crossed with male *Drosophila* carrying certain *UAS-X* transgenes. In the F1 progeny flies, *trol-GAL4* will induce the expression of the *UAS-X* transgene in the ocellar region. With the help of dominant genetic markers on balancer chromosomes, I can identify F1 adult flies with the correct genotype (containing both *trol-GAL4* and *UAS-X*). Using dissecting microscope, I then checked these adults to see if their ocelli has any changes. The ocellar phenotypes were photographed under light microscope (Zeiss Axioplan 2). Several partially focused images were taken. Then the software Helicon Focus was operated to create one completely focused image from the focused areas

(A)



The flies without balancer was picked.

(B) Dominant marker for Balancer Chromosome

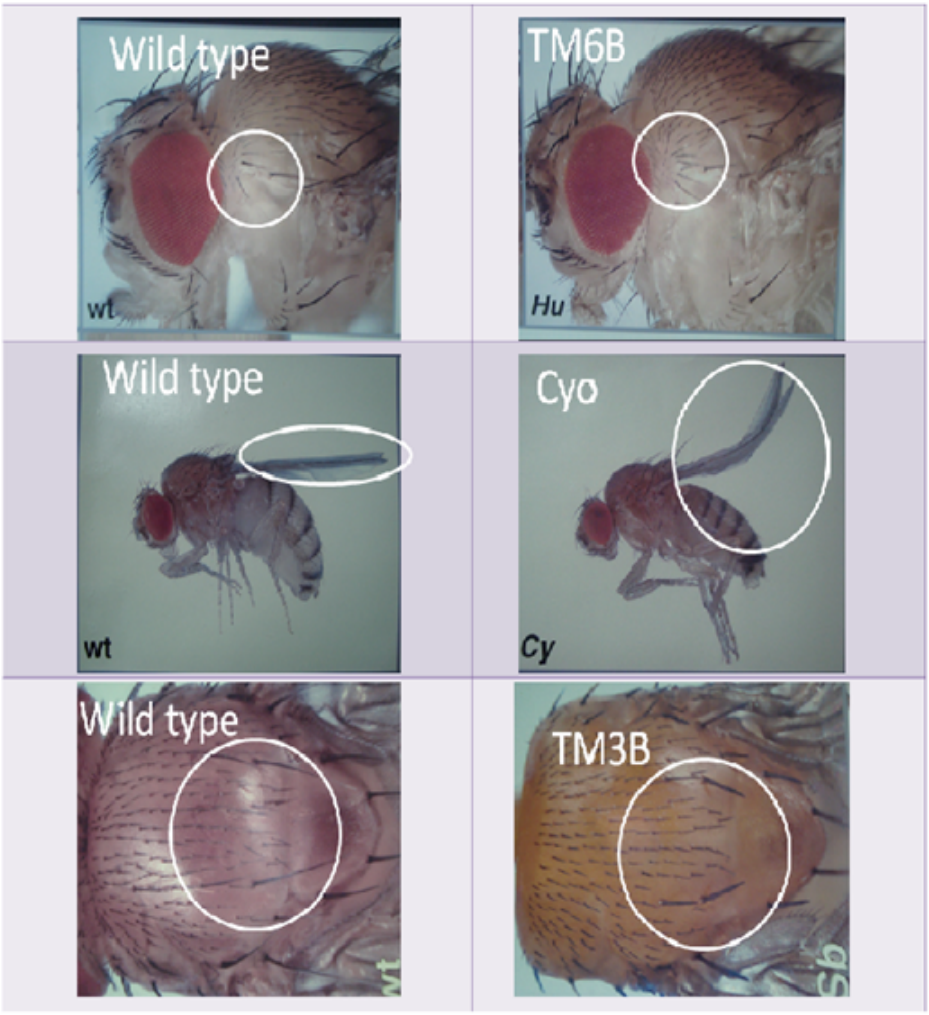


Fig. 6 The experimental procedures to pick the flies with specific genes

(A) To use the UAS-GAL4 system to induce gene expression in the ocellar region, the *trol-GAL4* females were mated with *UAS-X* males. The F1 progeny were screened for ocellar phenotype.

(B) Several dominant genetic markers on the balancer chromosome were used in the study in order to identify the flies with the correct genotype of *trol-GAL4* + *UAS-X*. The dominant markers are: *Tubby* (*Tb*) and *Humeral* (*Hu*) for *TM6B*, *Curly* (*Cy*) on *CyO*, and *Stubble* (*Sb*) on *TM3B*.

(B) Dissection of eye-antenna discs from larva

Dissection was performed to pull two eye-antenna discs out of a late third instar larva. The eye-antenna disc is a part of the larva that will turn into antenna and visual system including compound eyes and ocelli during metamorphosis (pupal-adult transformation).

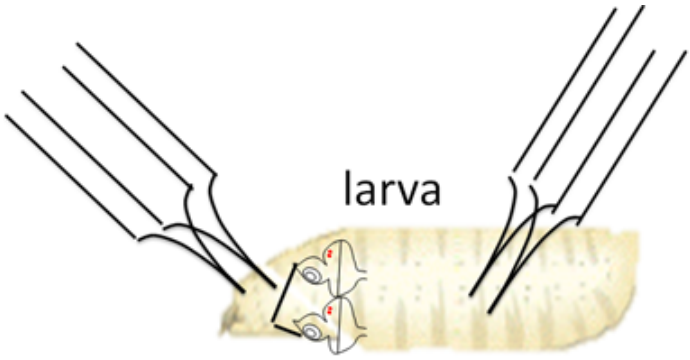


Fig. 7 larva dissection

(C) Immunostaining

The dissected eye-antenna discs were fixed by 4% paraformaldehyde for 20-30 minutes. The fixed samples were washed with PBST (PBS with 0.3% Trition X-100) for 10 minutes for 3 times. Then the primary antibody will be added into the sample and the target protein in the eye discs will be bound by the first antibody. After adding the primary antibody, the sample were put into an incubator at 4°C overnight. The samples were washed with PBST for 10 minutes for 3 times. The next step is to use the secondary antibody to detect the primary antibody. The secondary antibody will bind to the primary antibody. Because these kinds of antibodies have been combined with fluorescent dyes, when they bind to the primary antibody, the whole complex can show fluorescence. The samples were mounted by 80% glycerol. and examined by confocal fluorescence microscope (Fig. 8).

Primary antibodies were used at the following diultions: mouse anti-Eya (1:200)(Iowa Developmental Studies Hybridoma Bank; IDSHB), Rat anti-Elav(1:500)(IDSHB). The primary antibodies were detected by fluorescent-conjugated secondary antibodies including donkey anti-RAT Dylight 649(1:200) (Jackson ImmunoResearch Laboratories), donkey anti mouse eya

Dylight 549 (1:200)(Jackson ImmunoResearch Laboratories). The nuclear were stained by 4',6-diamidino-2-phenylindole (DAPI) (1:500, stock concentration is 1 mg/ml).

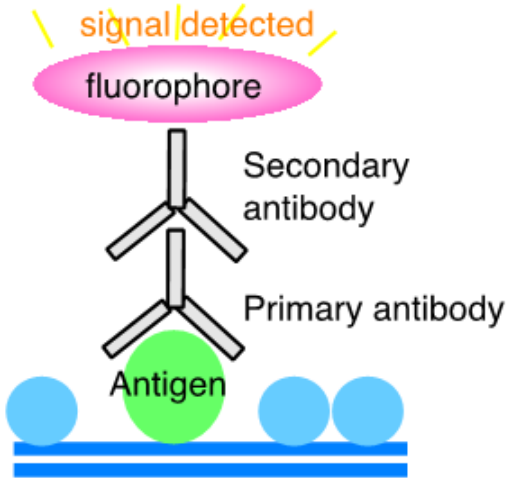


Fig. 8 Immunohistochemistry

Immunostaining was used in the study to examine the ocelli development in the larva stage of *Drosophila*. The immunostaining involved two stages: parimary and secondary antibody.

(D) Electroretinogram (ERG)

Electroretinogram (ERG) was used to measure electrical activity of ocellar photoreceptors in response to light. The ERG records the voltage difference between an active electrode placed in the ocelli and a ground electrode placed in the dorsal region of the head, and stimulation was given by a 1 second of bright white light illumination (Fig. 9).

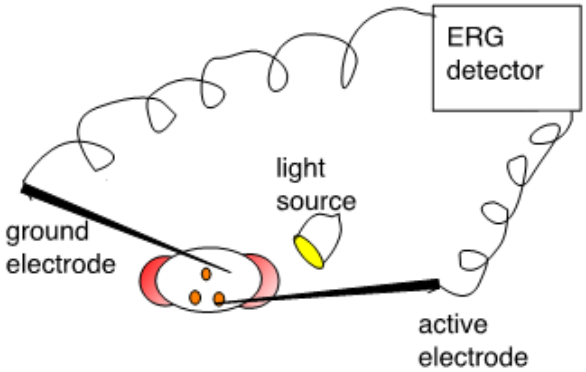
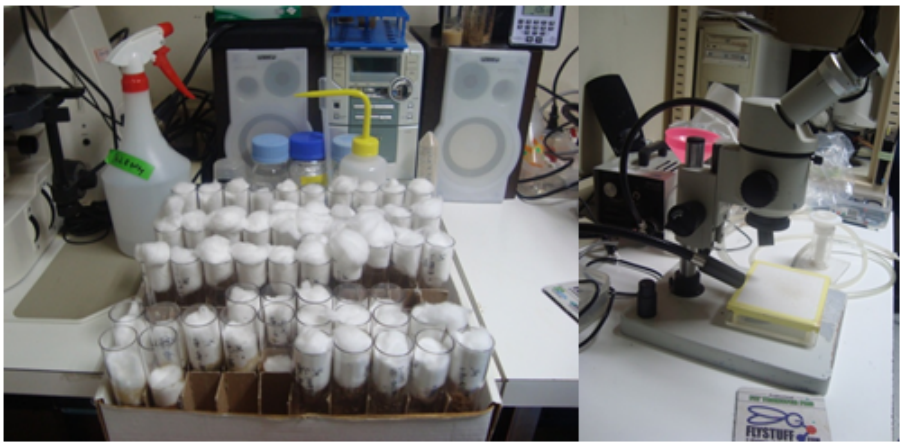


Fig. 9 Schematic of the placement of ground and recording electrode in the head and ocelli

(E)Materials and equipments

The materials of this experiment are *Drosophila*, compound microscope, dissecting microscope, photographing equipments, confocal microscope and the culturing tube.



The Drosophila culturing tubes　　The observing stage of Drosophila

Dissecting microscope (with camera)　　optical microscope (with camera)

Fig. 10 Experimental equipments
Dissecting microscope Nikon SMZ 1000 with digital camera Nikon D90 and optical microscope Zeiss Axioplan 2 with digital camera Nikon D90.

C. Results

(A) The genes being screened in order to identify the ocellar growth

genotype
trol>toy(III)
trol>toy(II)
trol>Toy
trol>tsh+toy (w;UAS-tsh/Cyo;UAS-toy/TM6B) <*>
trol>toy + tsh (UAS-toy/Sp;UAS-tsh/TM3 Sb eSer) <*>
trol>tsh+toy (w;UAS-tsh/Cyo;UAS-toy/TM6B)
trol>ey (III)
trol>ey(II)
trol>eya+so
trol>eya
trol>dpp+eya
trol>eyg
trol>ey+omb
trol>eyg(II)
trol>so+eya
trol>eyg-1+hth[12]
trol>Cyo/UAS-eyg-1+hth[12]
trol>hth[12]
trol>hth[15];TM3,Sb,Ser/UE10
trol>hth[15];UE10
trol>hth(III)
trol>Dll+hth
trol>tsh+hth
trol>hth[15]+p-35
trol>flu-arm+hth[12]

Table 1A

genotype
trol>N act
trol>btl(III)
trol>bnl
trol>ths-RNAi(kk)(II)
trol>bs-RNAi(kk) (II)
trol>pnt-RNAi(kk) (II)
trol>pnt p1
trol>sty-RNAi
trol>pnt p2
trol>ras
trol>dys.J
trol>upd(II)
trol>Grim(II)
trol>htl-RNAi
trol>htl.DN;htl.DN

Table 1B

genotype
trol>ras^{N17} (x)
trol>raf[1.gof(x)
trol>SEM
trol>ras1[wt]
trol>ras1[v12] (trol>ras1[v12]/Cyo(A-1))
trol>ras1[v12] (trol>ras1[v12]/TM3)
trol>ras^{N17}/Cyo(II)

Table 1C

genotype
trol>cdc^{x12}
trol>Rho^{N19}
trol>Rec^{N17}
trol>cdc 42^{N17}
trol>Rho^{N14}
trol>DIAP1
trol>p35
trol>ras^{N17} (III)
trol>Egfr^{DN};Egfr^{DN}
trol>Egfr (λ top 4.2)
trol>λ-btl
trol>Gal 80^{ts};DN-btl
trol>-btl trol>htl.DN;htl.DN
trol>hth^{15};p35

Table 1D

genotype
trol>yw,ey [UE11]+dpp[293]
trol>w,hh[M1]+UE10
trol>wg+dTCF ΔN
trol>tsh+N
trol>w,tsh+N[DN-6]
trol>w,tsh+N$^{[act]}$
trol>w,EYG-VP16+N[act]
trol>w,omb[4-15]+hth[12]
trol>w,hth[15]+omb[2-17]
trol>w,omb+Dll
trol>ey+omb
trol>omb[4-15](II)+dTCF[DN](III)
trol>w,wg
trol>Dll+hth

Table 1E

Table1 The genes that are screened in order to identify the ocellar developmental genes.

The genes that are marked with yellow color are the genes which were found to influence the ocelli phenotype. (The Cyo, TM6B, TM3 Sb Ser shown in the table is balancer chromosome, its goal is to help distinguishing the genotype of progency..

Because the ocelli and compound eyes arise from the same imaginal disc, I assume that the genes that affect the growth of compound eyes might have some interactions with ocelli too. Therefore I took the genes listed in table 1A to do the first stage of screening.

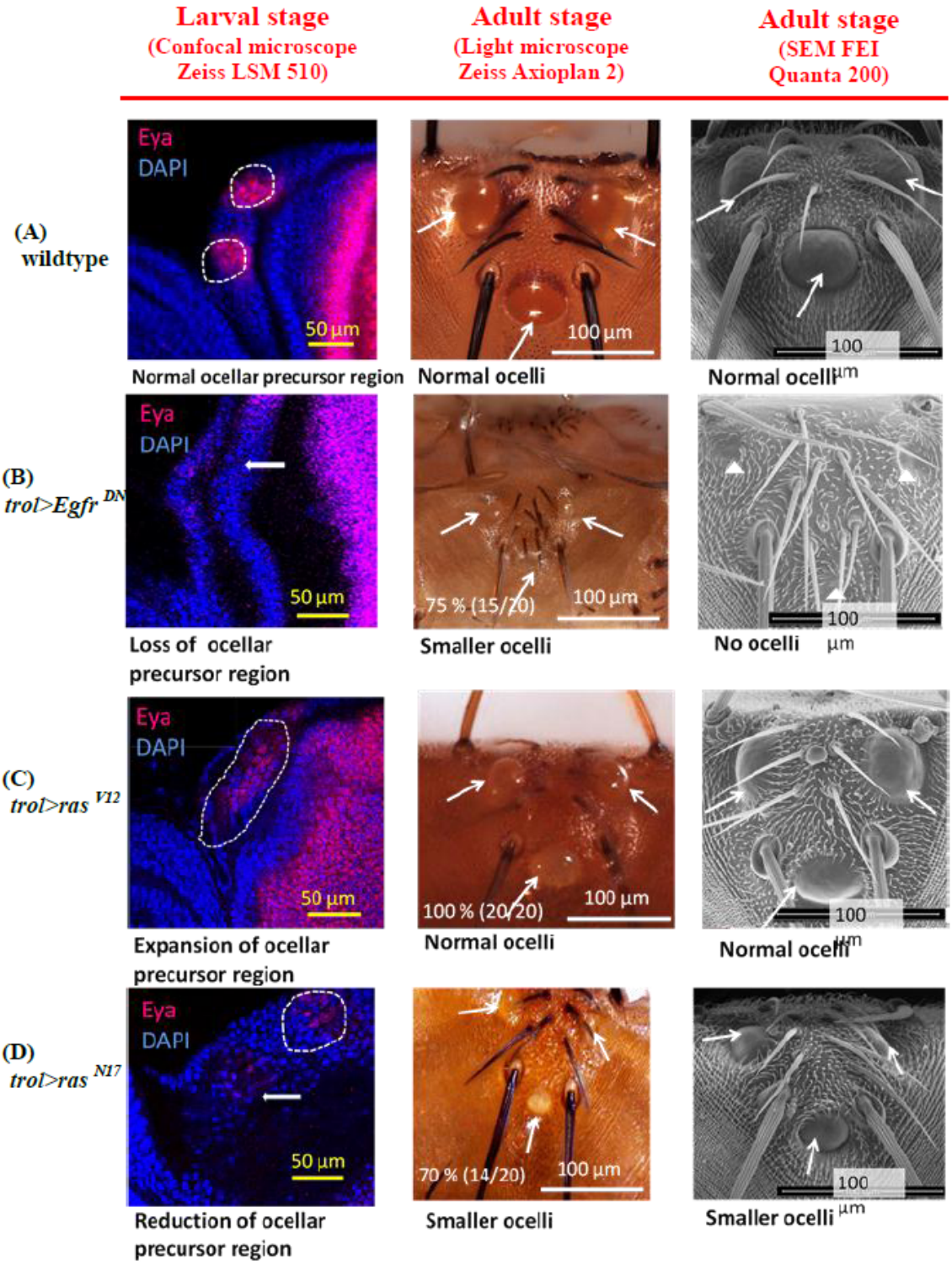
In the meanwhile of screening for developmental genes of compound eyes whether have an influence on the ocellar development, I took several genes that are crucial for Drosophila development to test if the genes affect the development of ocelli (Table 1B)

After discovering the important role that *ras* plays on the ocellar development, I took the dominant negative form, constitutive active form and different level of dominant negative form of *ras* to probe into its effects and mechanism on the ocellar development (Table 1C).

Furthermore, I would like to search for the upstream and downstream gene of *ras* and build a signaling pathway of ocellar development, so I took several upstream genes that are known to regulate *ras* signaling and also several downstream genes of *ras* (Table 1D).

Some other genes are also screened to test if they are also important In ocellar development (Table 1E)

(B) Egfr-Ras pathway was identified to be essential for the development of oceli



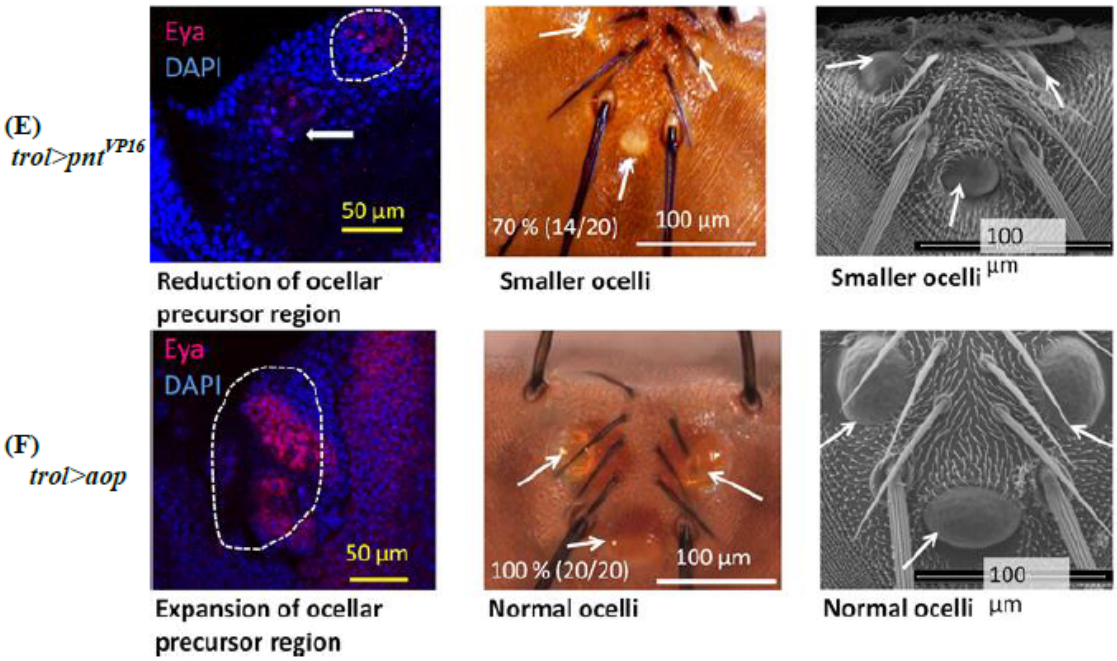


Fig. 11 Expression of eya in eye-antenna disc and eye morphology in adult flies

(A) Wild type

(B) *trol>Egfr*^{DN}, a dominant negative form of *Egfr* :
In larval stage, it shows the loss of ocellar region. In adult *Drosophila*, Some of the mutant flies' ocelli are much smaller, while the others' ocelli seemed to be missing.

(C) *trol>ras*^{V12}, a constitutive active form of *ras* :
It exhibits expanded Eya staining area in eye-antenna disc, and normal ocelli in adult.

(D) *trol>ras*^{N17}, a dominant negative form of *ras* :
In larval stage, it demonstrates the expanded Eya staining area. In adult Drosophila, three ocelli are present but decrease dramatically in their size under SEM.
(The diameter of the medial ocellus dropped from 46.4 µm to 28.6 µm.)

(E) *trol> pointed*^{VP16}, a constitutive active form of *pointed* :
It shows expanded Eya staining area in eye-antenna disc, and normal ocelli in adult.

(F) *trol>aop*, an inhibitory transcriptional factor
It exhibits loss of Eya staining area in eye-antenna disc, and normal ocelli in adult.

(C) Electroretinogram (ERG) response to light stimulation of ocelli

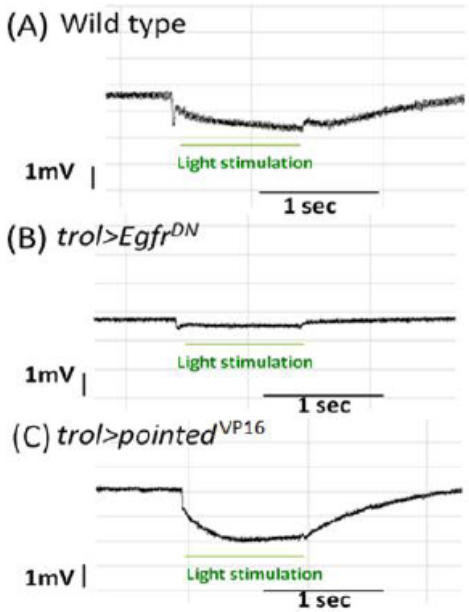


Fig. 12 The Electroretinogram (ERG) response of ocelli in

(A) Wild type

(B) ocellar mutant (*trol>Egfr*^{DN})

(C) Ocellar mutant (*trol>pointed*^{VP16})

The ERG is measured by the voltage difference between an active electrode and a ground electrode. The results showed that compared to ocelli of wild type, the mutant ocelli of *trol>Egfr*^{DN} showed little or no voltage change in response to light stimulation. In contrast, the mutant ocelli of *trol>pointed*^{VP16} demonstrated stronger voltage change in response to light stimulation.

(D) Electroretinogram (ERG) response to light stimulation of compound eyes

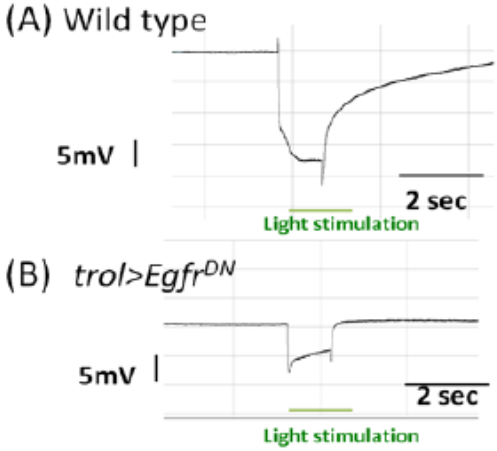


Fig. 13 The Electroretinogram (ERG) response of compound eyes in

(A) Wild type

(B) Ocellar mutant (*trol>Egfr*^{DN})

The ERG of ocellar mutant's compund eyes was measured to see if the mutant ocelli would influence the ERG response of compound eyes. The result showed that the ocellar mutant exhibited smaller voltage amplitude.

(E) Results of overexpression of *eyg+hth*

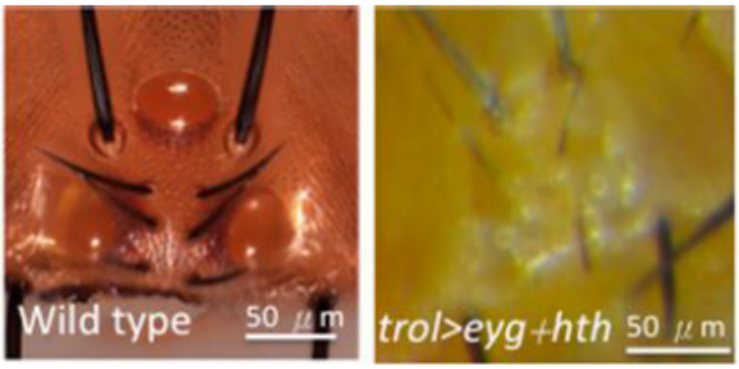


Fig. 14 The phenotype change of ocelli when co-expressing *eyg* and *hth*
Three ocelli turn into several small ocelli

(F) Results of overexpression of *eyg+ser*

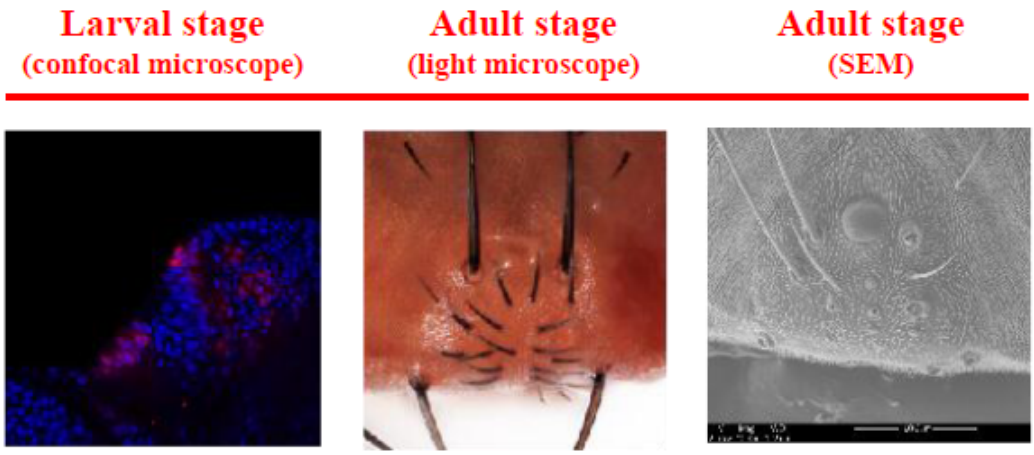


Fig. 15 The phenotype change of ocelli when overexpressing eyg+ser

Some of the mutants' only own the medial ocellus, while some of the mutants have three ocelli but except the medial ocellus, other two lateral ocelli were dramatically reduced in size.

D. Discussion and Conclusions

(A) Discussion

In the experiment, we identified some genes which might influence the development of ocelli, including Egfr-Ras signaling pathway (*Egfr, ras, pointed, aop*), *eyegone(eyg)* with *homothorax(hth)* and eyg with serrate (ser).

1. Mechanism of Egfr-Ras pathway

Egfr-Ras pathway were found to be crucial for ocellar development. Blocking of *Egfr-Ras* signaling pathway by (1) overexpression of $Egfr^{DN}$, the dominant negative form of *Egfr* (Fig. 11B) and (2) Ras^{N17}, the dominant negative form of *ras* (Fig. 11D) resulted in reduced ocellar precursor region in larva eye disc and adult ocelli size are also reduced.

In contrast, enhancing *Egfr-Ras* signaling pathway by (1) $pointed^{VP16,}$ the constitutive active form of *pointed* (Fig. 11E) and (2) ras^{V12}, the constitutive active form of *ras* (Fig. 11C) resulted in expansion of ocellar precursor region in larva eye disc. Because ras is known as a downstream component of Egfr and aop/pointed is known as a downstream component of ras, it is assumed that Egfr-Ras pathway is essential for ocellar development.

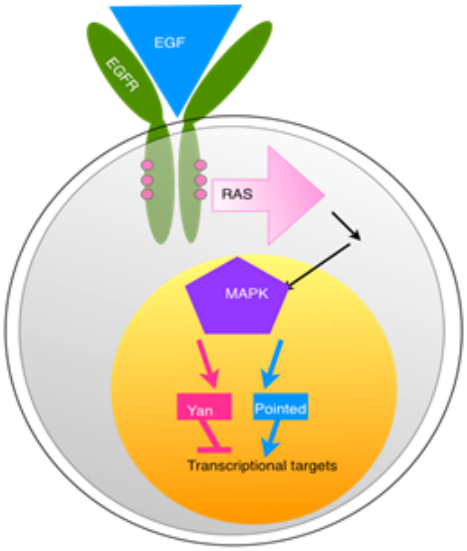


Fig. 18 Working model of EGFR/ RAS signaling on ocellar development
From the data of the experiment, a signaling pathway of ocellar development is being proposed.

2. Hypothetical interaction between gene so and *Egfr-Ras* pathway

Overexpression the pointed^{vp16} or Ras^{V12} cause the expansion of ocellar precursor region, but the adult ocellar phenotype still normal. It imply that other gene (gene X) may also involved in the ocellar development. If we co-expression of eya and so resulted in the enlargement of ocellar region in adult (Fig.19B). These suggest that the gene X might be *so*, which may also influence the ocelli development (Fig. 19A).

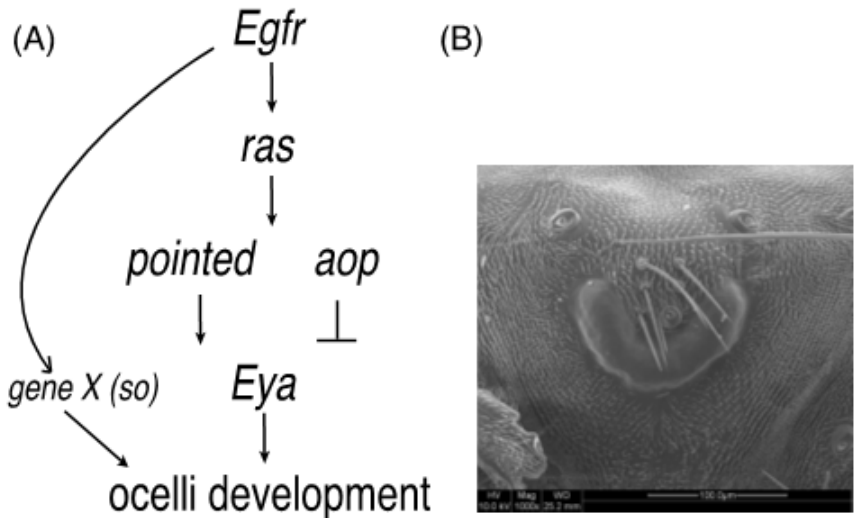


Fig. 19 Putative interaction between so and *Egfr-Ras* pathway
(A): The gene interaction pathway between gene x and Egfr-Ras pathway
(B): trol- GAL4 drives the co-expression of *eya* and *so (trol>eya+so)*

3. Possible reason for dramatic lower Electroretinogram (ERG) response to light stimulation of ocellar mutant's compound eyes

The compound eyes of ocellar mutant flies (*trol>Egfr*^{DN}) showed dramatically weaker electroretinogram (ERG) response to light stimulation (Fig. 12). It is known that ocelli would send signal to the lobular or lamina neurons, these neurons can adjust the light that compound eyes received. Also if the ocelli were covered, the ERG of the compound eyes would reduce up to 20% (Rence et al 1988).

Nevertheless, the ERG of the ocellar mutant's compound eyes was reduced about 60% in this experiment. These suggest the ocelli is important for normal function of compound eye.

4. Hypothetical interaction between *eyg* and *hth*

We found out that the co-expression of eyegone (eyg) and homothorax (hth)may cause more small ocelli, but do not transform the ocelli to compound eyes (Fig. 14). We presume it is because that eyg will induce the MF-initiation; while hthinhibits the MF-initiation, so their actions cancel each other. eyg may induce cell proliferation, leading to more ocelli.

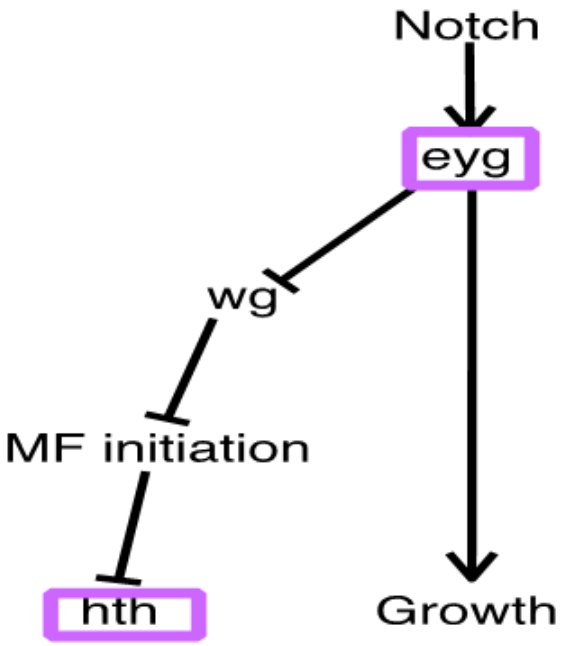


Fig. 20 Hypothesis for eyg and hth function on the development of ocelli
When eyg and hth are being coexpressed, it leads to the growth of small ocelli. It is assumed the eyg will trigger the MF initiation (a mechanism that involved in compound eye development) by inhibiting wg, while hth will inhibit MF initiation. Therefore the result of ocelli turning into compound eyes wasn't observed. Nevertheless, eyg may induce cell proliferation, leading to the the appearance of small ocelli.

5. Speculative Mechanism of the co-expression of *eyegone (eyg)* and *Serrate (Ser)*

When *eyg* and *Ser* were being co-expressed, it caused some of the mutants' only own the medial ocellus, while some of the mutants have three ocelli but except the medial ocellus, other two lateral ocelli were dramatically reduced in size (Fig. 15). From the expression pattern of *eyes absent (eya)* in larval stage, we can see that in per eye-disc, the ocellar precursor region only has one normal ocellar precursor region. In the other ocellar precursor region, it becomes several small ocellar precursor regions. The several small ocellar regions could not successfully developed into ocelli during metamorphosis. Only the medial ocellus which formed from the merging of two eye discs survival, because in the larval stage the

normal ocellar precursor region is the place where is responsible for the development of medial ocellus.

(B) Conclusions

We found several genes that are important for ocellar development in *Drosophila*. Blocking of Egfr-Ras signaling pathway resulted in reduced ocellar precursor region in larva eye disc and adult ocelli size are also reduced. In contrast, enhancing Egfr-Ras signaling pathway resulted in expansion of ocellar precursor region in larva eye disc. However, the size of ocelli in adult flies still remains normal. These suggest Egfr-Ras signaling is required but not sufficient for ocellar development. In the ERG experiment, we found that Egfr-Ras signaling not only affect ocellar development, but also normal ocellar function.

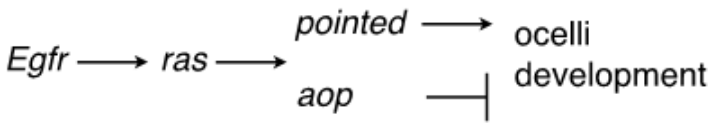


Fig. 21 Egfr-Ras pathway for ocellar development

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評語

1. 此研究利用基因轉植果蠅及遺傳學方法找出控制果蠅單眼發育的新基因(Ras)，其研究成果頗有新穎性。

2. 此研究亦證明 EGFR 為 Ras 的上游基因，因此值得繼續探討 Ras 基因在單眼發育的實際功能。

3. 另外亦發現其他單眼調控基因，整體而言，此研究對單眼發育有不錯的貢獻。